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<b>(21) International Application Number:</b> PCT/AU94/00740 <b>(22) International Filing Date:</b> 30 November 1994 (30.11.94)  <b>(30) Priority Data:</b> PM 2705 30 November 1993 (30.11.93) AU PM 8234 16 September 1994 (16.09.94) AU  <b>(71) Applicant (for all designated States except US):</b> THE UNIVERSITY OF QUEENSLAND [AU/AU]; St. Lucia, QLD 4072 (AU).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MORTON, Halle [AU/AU]; 75 Orion Street, Coorparoo, QLD 4151 (AU). CAVANAGH, Alice, Christina [AU/AU]; 29 Anderson Avenue, Ashgrove, QLD 4060 (AU).  <b>(74) Agent:</b> KELLY, Robin, Thomas; Fisher & Kelly, Level One, 349 Coronation Drive, Milton, QLD 4064 (AU).	<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> CHAPERONIN 10  <b>(57) Abstract</b>  A process for the detection of cpn10 in serum or other biological fluids including the steps of (i) raising antibody to cpn10; (ii) reacting said antibody with a sample of biological fluid suspected of containing cpn10; and (iii) detecting the presence of cpn10 in said sample by a signal amplification resulting from production of a cpn10-antibody complex. There is also provided a process for promotion of cell growth or immunosuppression including the step of administration of cpn10 to a mammalian subject. There is also provided recombinant cpn10 having the sequence GSAGQAFRKFLPLFDRVLVERSAETVTKGGIMLPEKSQGVQLQATVEAVGSGSKGKGGEIQPVSVKEGDKVLLPEYGGTKVVLDDKDYFLFRDGDILGKYVD.		

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TITLE

"CHAPERONIN 10"

FIELD OF THE INVENTION

THIS INVENTION relates to chaperonin 10 otherwise known as

5 cpn10.

PRIOR ART

Chaperonins belong to a wider class of molecular chaperones, molecules involved in post-translational folding, targeting and assembly of other proteins, but which do not themselves form part of the final assembled structure as discussed by Ellis *et al.*, 1991, *Annu. Rev. Biochem.* 60 321-347. Most molecular chaperones are "heat shock" or "stress" proteins (hsp); i.e. their production is induced or increased by a variety of cellular insults (such as metabolic disruption, oxygen radicals, inflammation, infection and transformation), heat being only one of the better studies stresses as reviewed by Lindquist *et al.*, 1988, *Annu. Rev. Genet.* 22 631-677. As well as these quantitative changes in specific protein levels, stress can induce the movement of constitutively produced stress proteins to different cellular compartments as referred to in the Lindquist reference mentioned above. The heat shock response is one of the most highly conserved genetic system known and the various heat shock protein families are among the most evolutionarily stable proteins in existence. As well as enabling cells to cope under adverse conditions, members of these families perform essential functions in normal cells.

There are two types of cpn molecules, cpn60 (monomeric  $M_r$  ~60 000) and cpn10 (monomeric  $M_r$  ~10 000). Cpn60 has been studied extensively. It has been identified in all bacteria, mitochondria and plastids examined, and a cytoplasmic form, TCP-1, has been identified in eukaryotic cells; its presence on the surface of some cells has been reported, although this has been questioned in the Ellis reference referred to above and also in van Eden, 1991, *Immunol. Reviews* 121 5-28. Until very recently, cpn10 had been identified only in bacteria but structural and functional equivalents have now been found in chloroplasts (Bertsch *et al.*, 1992, *Proceedings of the*

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National Academy of Sciences USA 89 8696-8700) and in rat (Hartman *et al.*, 1992, Proceedings of the National Academy of Sciences USA 89 3394-3398) and bovine liver mitochondria (Lubben *et al.*, 1990, Proceedings of the National Academy of Sciences USA 87 7683-7687).

5 Cpn60 and cpn10 interact functionally, in the presence of ATP, to mediate protein assembly. Instances of cpn10 acting independently of cpn60 have not yet been reported but cpn60, apparently acting alone, has been implicated in quite disparate events. For example, it is an immuno-dominant target of both antibody and T-cell responses during bacterial infections but, because the protein is so highly conserved, self reactivity is generated. Healthy individuals may use this self-recognition to eliminate transformed and infected autologous cells but defects in control of such recognition may lead to autoimmune disease as discussed by van Eden, 1991, Immunol. Reviews 121 5-28. Not surprisingly, cpn60 has been associated with conditions such as rheumatoid arthritis. There is thus a growing awareness that molecular chaperones, with their capacity to bind to and alter the conformation of a wide variety of polypeptides, may occupy key roles in cellular functions other than protein biogenesis. Reference may also be made to Hartman *et al.*, 1993, Proceedings of the National Academy of Sciences USA 90 2276-2280 which describes the stabilization of protein molecules using cpn10 and cpn60.

20 It can also be established that for mammalian cpn10's, there is a very close sequence homology. Thus, for example, the rat cpn10 molecule (Hartman *et al.*, 1992) has greater than 99% homology with the structure of bovine cpn10 reported in EMBL Data Base Directory under MT BTC PN10 which was submitted by J. E. Walker, MRC Lab. of Molecular Biology, Hills Road, Cambridge, UK. This has to be contrasted with bacterial cpn10's which have an average degree of homology with rat chaperonin 10 of only 34% (Hartman *et al.*, 1992).

#### EARLY PREGNANCY FACTOR (EPF)

30 EPF was first described as a pregnancy associated substance (Morton *et al.*, 1976, Proc. R. Soc. B. 193 413-419) and its discovery created considerable interest as it enabled the detection of a potential pregnancy within

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6-24 hours of fertilisation. Initially EPF was assigned a role as an immuno-suppressant by virtue of its ability to release suppressor factors from lymphocytes (Rolfe *et al.*, 1988, Clin. exp. Immunol. 73 219-225). These suppressor factors depress the delayed type hypersensitivity reaction in mice and therefore might suppress a possible maternal immune response against the antigenically alien fetus. More recent studies have shown that production of EPF is not confined to pregnancy. It is a product of primary and neoplastic cell proliferation and under these conditions acts as a growth factor (Quinn *et al.*, 1990, Clin. exp. Immunol. 80 100-108; Cancer Immunol. Immunother, 1992, 34 265-271). EPF is also a product of platelet activation and it is proposed therefore that it may play a part in wound healing and skin repair (Cavanagh *et al.*, 1991, Journal Reproduction and Fertility 93, 355-365).

EPF is reviewed in detail by Morton *et al.*, 1992, Early Pregnancy Factor, Seminars in Reproductive Endocrinology 10 72-82. The site and regulation of EPF production is described, followed by the purification of EPF from platelets and the relationship of the purified product to EPF derived from other sources. This review also discusses certain aspects of the bioassay for EPF (i.e. the rosette inhibition test) including monitoring purification procedures and investigating sources of production. The biological activity of EPF is discussed and possible clinical applications of EPF and its antagonists are described.

Morton *et al.*, 1992, Reprod. Fertil Dev. 4 411-422 reviews previous publications describing the immuno suppressive and growth factor properties of EPF. The role of EPF in maintaining the pre-embryo is also discussed in this reference.

To date, the rosette inhibition test remains the only means of detecting EPF in complex biological mixtures (Morton *et al.*, 1976, Proc R Soc B 413-419). This assay is dependent on the original finding of Bach and Antoine, 1968, Nature (Lond) 217 658-659 that an immunosuppressive anti-lymphocyte serum (ALS) can inhibit spontaneous rosette formation *in vitro* between lymphocytes and heterologous red blood cells. A modification of the assay was introduced to detect EPF after it was demonstrated that

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lymphocytes, preincubated in EPF, give a significantly higher rosette inhibition titre (RIT) with an ALS than do lymphocytes from the same donor without EPF as described in the 1976 reference above. This test has been described in detail in the above 1976 reference as well as in Morton *et al.*, 1987, in "In Current Topics in Developmental Biology" Vol 23 73-92, Academic Press, San Diego, but briefly it involves a cascade of events with EPF binding to lymphocytes and sequentially inducing the release of suppressor factors (Rolfe *et al.*, 1988, Clin. exp. Immunol. 73 219-225); (Rolfe *et al.*, 1989, Immunol. Cell Biol. 67 205-208).

10 In Athanasas-Platsis *et al.*, 1989, Journal Reproduction and Fertility 87 495-502 and Athanasas-Platsis *et al.*, 1991, Journal Reproduction and Fertility 92 443-451, there is described the production of monoclonal and polyclonal antibodies to EPF and passive immunization of pregnant mice with these antibodies which causes loss of embryonic viability. These studies established that EPF is necessary for the successful establishment of pregnancy.

In Quinn *et al.*, 1990, Clin. exp. Immunol. 80 100-108, it is proposed that EPF is a growth regulated product of cultured tumour and transformed cells. These cells are also dependent upon EPF for continued growth i.e. EPF acts in an autocrine mode.

It has been established that EPF plays a role in promoting tumour growth since the growth of tumour cells can be significantly retarded by anti-EPF mAbs. In addition this reference suggests that hybridomas producing high affinity anti-EPF antibodies may be inherently unstable.

25 In Quinn *et al.*, 1992, Cancer Immunol. Immunother. 34 265-271, there is also described the effect of monoclonal antibodies (mAbs) to EPF on the *in vivo* growth of transplantable murine tumours. The main thrust of this reference is that neutralisation of EPF retards tumour growth *in vivo*.

It is clear from the above Quinn *et al.* 1992 reference that when cancer is in the very early stage of growth, neutralisation of EPF by anti-EPF mAb will prevent its development. However, once the cancer becomes established, treatment with these mAbs will retard but not entirely destroy the

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tumour.

Other references in regard to the role of EPF in tumour growth include Quinn, 1991, Immunol. Cell Biol. 69 1-6 and Quinn, K. A. in a PhD thesis entitled "Early pregnancy factor: a novel factor involved in cell proliferation" from the University of Queensland in Australia in 1991.

From the foregoing it therefore will be appreciated that EPF is indicative of and necessary for embryonic well being and has immunosuppressive properties. Recent studies indicate the importance of EPF as a growth regulator of transformed, neoplastic and normal cells while its presence in platelets implies a role in inflammation and wound healing.

A molecule possessing this exceptional combination of properties has great significance but to date the structure of this molecule has proved to be elusive.

It has now been established unexpectedly that mammalian chaperonin 10 has an amino acid sequence which is the same as early pregnancy factor (EPF).

#### SUMMARY OF THE INVENTION

In one aspect, the invention resides in the discovery that cpn10 is EPF and has the hitherto unknown or unsuspected properties demonstrated by EPF. The unknown or unsuspected properties of cpn10 include extracellular activities such as the ability to act as a growth factor and an immunosuppressive factor. In another aspect the invention provides one or more methods for using cpn10 to exploit the unknown or unsuspected properties of cpn10. The one or more methods includes a method of using cpn10 to promote growth and a method of using cpn10 to suppress immunological activity.

The term "cpn10" as used herein includes within its scope eucaryotic cpn10 as well as procaryotic cpn10 inclusive of *groES* or derivatives of cpn10. The cpn10 may be purified from any biological source or produced synthetically or by recombinant DNA technology. The term also includes biological fragments.

The present invention also includes within its scope a modified

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recombinant as well as derivatives and peptide fragments derived therefrom.

The present invention includes within its scope the following.

1. ASSAY FOR CPN10

5 The detection of cpn10 in serum or other biological fluids using monoclonal or polyclonal antibodies against cpn10 or against modifications or fragments thereof alone or in combination with each other or with cpn60 (in the presence of ATP or other nucleotide triphosphates) for the purpose of:-

- (a) pregnancy diagnosis in any mammalian species;
- 10 (b) monitoring embryonic well-being in "at-risk" pregnancies;
- (c) diagnosis of tumours; and
- (d) monitoring patients after surgical removal of tumours.

2. TREATMENT WITH CPN10

15 The use of cpn10 as a growth factor or immunosuppressant in the treatment of:

- (a) skin or organ grafts;
- (b) wound healing, tissue repair or regeneration of tissue;
- (c) autoimmune disease;
- (d) infertility/miscarriage;
- 20 (e) allergic disease; and
- (f) inflammatory conditions.

EXPERIMENTAL

25 1. PURIFICATION OF CPN10

- (a) *Purification of Human EPF from Human Blood Platelets*  
(FIG. 1a, 1b, 1c, 1d)

Extraction

30 Platelet concentrates (from the Blood Bank), up to 7 days clinically outdated, were washed with Tyrodes buffer, following the techniques described in Methods in Enzymology, 1989, 169 7-11, snap frozen in liquid N<sub>2</sub> and stored at -70°C.



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Immediately prior to purification, approximately 100 washed platelet units were thawed in a boiling water bath, then held at 75-85°C for 15 min with continuous, gentle stirring. After cooling on ice, cellular debris was removed by centrifugation (8000 g, 20 min, 4°C) and the pellet extracted twice by homogenisation in 0.05 M-acetic acid/0.1 M-NaCl/0.1 mg/ml sodium azide pH 3.0 followed by centrifugation (8 000 g, 15 min 4°C). The three supernatants were pooled giving a total extract volume of 500-600 ml.

Ion-exchange chromatography

This extract from 100 platelet units was adjusted to pH 3.0 with conc. HCl and stirred gently, overnight, 4°C, with 250 ml SP-Sephadex C-25 (Pharmacia-LKB), previously swollen and equilibrated with 0.05 M-acetic acid/0.1 M-NaCl pH 3.0. The gel was then packed into a column washed with 20 vol of the same buffer and eluted with 5 vol 0.5 M-sodium phosphate buffer/0.05 M-NaCl pH 7.5. The gel was then discarded.

Affinity chromatography

The SP-Sephadex eluate was adjusted to pH 6.3-6.4 with conc. HCl and applied to a column of Heparin-Sepharose CL-6B (2.5 x 7.5cm; Pharmacia-LKB) previously equilibrated with 0.05 M-sodium phosphate buffer/0.05 M-NaCl pH 6.3. The column was then washed with 5 vol of the same buffer and eluted with 5 vol 0.05 M-Tris-HCl/5 mM-CaCl<sub>2</sub>/0.2 M-NaCl pH 7.5, applied in the reverse direction to that used for sample application.

High performance hydrophobic interaction chromatography (HIC-h.p.l.c.)

Solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the Heparin-Sepharose eluate to a final concentration of 2 M and, after passage through an 0.45 µm filter, the sample was pumped through a dedicated solvent line onto a TSK Phenyl 5PW column (7.5 x 75mm, Pharmacia-LKB), previously equilibrated with 0.1 M-Tris-HCl pH 7.0/5mM CaCl<sub>2</sub>/2 M-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The column was washed with 10 vol of the same buffer and eluted with a 50 min linear gradient from this buffer to 0.1 M-Tris-HCl pH 7.0/5 mM-CaCl<sub>2</sub>/10% acetonitrile. (FIG. 1a)

RP-h.p.l.c.-1

Active HIC-h.p.l.c. fractions were pooled, then fractionated on a C<sub>3</sub> column (Ultrapore RPSC, Beckman Instruments) using a solvent system

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consisting of A, 0.04 M Tris/HCl pH 7.0/5 mM-CaCl<sub>2</sub> and B, 0.04 M-Tris/HCl pH 7.0/5 mM-CaCl<sub>2</sub>/80% (v/v) acetonitrile. The column was equilibrated with Solvent A prior to sample application, after which it was washed with 5 vol solvent A and eluted with a 30 min linear gradient from this solvent to 75% solvent B. (FIG. 1b)

RP-h.p.l.c.2

Active fractions from RP-h.p.l.c.-1 of several 100 unit platelet preparations were pooled, EDTA and DTT added to a final concentration of 20mM and 1 mM respectively and the mixture allowed to stand for 0.5-1 h, 4°C. Following dilution with 2 vol solvent A, it was applied to a C<sub>3</sub> column, dedicated to this and subsequent steps, and fractionated as described for RP-h.p.l.c.-1, but omitting CaCl<sub>2</sub>. (FIG. 1c)

Rph.p.l.c.3

Active fractions from RP-h.p.l.c.-2 were pooled, trifluoroacetic acid (TFA) added to a final concentration of 0.1% and, following dilution with 2 vol 0.1% TFA, the mixture was applied to the C<sub>3</sub> column, which had been equilibrated previously with 0.1% TFA. The column was then eluted with a 30 min linear gradient from this solvent to 60% (v/v) acetonitrile/0.1% TFA, followed by a 3 min linear gradient to 90% (v/v) acetonitrile/0.1% TFA. Active fractions were pooled. (FIG. 1d)

One unit represents platelets from a single blood donation which is approximately 500 ml. The "active fractions" were fractions active in the rosette inhibition test.

Purification of EPF from other sources

EPF has been purified from various sources as discussed in Cavanagh & Morton, 1994, Eur. J. Biochem. 222 551-560; Quinn *et al.*, 1994, Hepatology 20 in press.

In all instances, biological activity followed the same pattern throughout the complex purification scheme described above for human platelets. Furthermore the final active fraction from all sources was bound specifically by an immobilised monoclonal anti-EPF and could be recovered virtually quantitatively (see FIG. 1e).

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These studies are important for several reasons:-

- A. The biochemical and immunological similarity observed with all these materials provides strong evidence that the bioassay is detecting a single substance or closely related family of substances acting in diverse biological situations.
- 5 B. The active agents purified from all of these materials are from several to many orders of magnitude more potent than virtually all of the substances previously reported to be EPF. This confirms our surmise, based on detailed analysis of the EPF bioassay as discussed above, that activity associated with most putative EPF preparations must reflect the presence of a very minor contaminant.
- 10 C. The only source materials providing sufficient EPF to study at the protein (as opposed to activity) level were platelets and regenerating liver, yielding, respectively, an average of 15  $\mu\text{g}$  per 100 units (equivalent to  $\sim 50$  litre blood) and 5  $\mu\text{g}$  per 40 g tissue (liver remnant from 6 rats). It is immediately apparent that far more EPF is present within the cell than appears in the extracellular space; nevertheless, accumulated knowledge of the biology of EPF (reviewed recently in the abovementioned Morton *et al.* 1992 reference) indicates that this extracellular appearance is not fortuitous.
- 15 20

Human platelet-derived EPF, being most abundant, has been studied in some detail. On SDS-PAGE, it ran as a single band of  $M_r$  approx. 8.500, coincident with biological activity (see FIG. 2a); EPF from regenerating rat liver exhibited identical behaviour. Mass spectrometry of the platelet material provided an accurate and precise determination of molecular mass  $10\,843.5 \pm 2$  Da, along with definitive evidence of the high degree of homogeneity of the preparation (see FIG. 2b). Following attempts at Edman degradation, which indicated that the molecule is N-blocked, proteolytic cleavage of approx. 4 nmol EPF was undertaken. Resultant peptide fragments were separated by reversed-phase HPLC and subjected to sequencing by

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Edman degradation. Three areas of sequence containing 12 (fragment 1), 27 (fragment 2) and 33 (fragment 3) residues were found to correspond with residues 7 to 18, 27 - 53 and 69 - 101 (the C-terminus) in rat mitochondrial cpn10. In fragment 2, residue 52 was different (S in cpn10, G in rat cpn10; this change alone could account for human cpn10 being 30 Da larger than rat cpn10). All other residues were identical, consistent with the highly conserved nature of chaperonins (see FIG. 2c).

Since confirming sequence identity between EPF and cpn10, several studies of functional relationship have been performed, using rat mitochondrial cpn10, *E. coli* cpn10 (known as *groES*) and *E. coli* cpn60 (*groEL*). First it has been demonstrated that cpn10 can act as EPF. Rat cpn10 was tested in the EPF bioassay and found to be positive over the range of dilutions expected; this activity could be neutralised by monoclonal antibodies to EPF (see TABLE 1). Interestingly, *E. coli* cpn10, which is ~ 40% homologous with rat cpn10, exhibited no activity in the bioassay (see TABLE 1); this is consistent with the observation that *E. coli* conditioned medium is not active in the EPF bioassay, while medium conditioned by all mammalian cell lines tested, as well as by yeast cells is active. Cpn60 was inactive in the bioassay and had no effect upon the activity of EPF. It was then shown that EPF can act as cpn10. EPF was mixed with cpn60, in the presence or absence of ATP, and the mixture fractionated on a TSK G3000SW gel permeation column; resultant fractions were analysed by SDS-PAGE. Cpn60 is a decatetramer and elutes in the excluded volume of this column (exclusion limit 300 000). In the presence of ATP, but not in its absence, EPF also appears in this fraction, demonstrating formation of a stable complex with cpn60. This fraction was active in the EPF bioassay but the equivalent fraction from the experiment without ATP (where EPF did not associate with cpn60) was not (see FIG. 3a). Thus EPF and cpn10 activity reside in the same molecule.

These investigations provide unequivocal evidence that platelet-derived EPF is a structural and functional homologue of cpn10; the relationship between cpn10 and activity in the rosette inhibition test was then

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examined (FIG. 3b). In the presence, but not in the absence of ATP, immobilised cpn60 could remove all activity from the archetypal source material, pregnancy serum and activity could be recovered by removing ATP from the immobilised complex. As with the experiment described in FIG. 3a, this requirement for ATP demonstrates the specificity of the interaction between cpn60 and the active moiety; cpn10 is thus the molecular entity initiating response in the EPF bioassay.

Identification of EPF as a cpn10 has been a major step forward in research on this subject and helps to explain many of the findings that have been made to date. Criticism has been raised against claims that EPF production occurs in such a wide variety of biological situations e.g. pre-and post-implantation pregnancy, primary and tumour cell proliferation and platelet activation. In its role as a hsp (heat stress protein), these are all conditions in which the rapid onset of EPF production would be expected. Functions of hsp's that are vital to the survival of cells are intracellular as shown in the Linquist *et al.* reference above. In contrast, the activity of EPF described to date is extracellular; for example, it appears in serum of mice within 4 to 6 hours after mating as discussed in Morton *et al.*, 1987, Current Topics in Development Biology, Vol 23 73-92 and 4 to 8 hours after partial hepatectomy in rats as shown in the Quinn PhD thesis (1991). We have shown that EPF can act in an autocrine mode as discussed in the Quinn *et al.*, 1990 reference referred to above or exocrine mode as discussed in the Rolfe *et al.* 1988 referred to above; these are not roles previously described for hsp's.

It will also be appreciated that since the structure of EPF is now known, it can be produced in commercial quantities by any suitable technique such as by recombinant DNA technology or chemical synthesis.

(b) *Cloning of human cDNA encoding cpn10 and production of cpn10*

Production for commercial use may be obtained by inserting a mammalian cpn10 gene, preferably a human cDNA cpn10 gene, into a suitable vector such as plasmids from the pGEX system, and pET system expressing the encoded mammalian cpn10 and purifying the recombinant cpn10.

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**Abbreviations**

	ANGIS	Australian National Genomic Information Service
	bp	base pair
	BSA	bovine serum albumin
5	cDNA	complementary DNA
	cpn10	Chaperonin 10
	DNA	deoxyribonucleic acid
	<i>E. coli</i>	<i>Escherichia coli</i>
	GSH	glutathione (reduced form)
10	GST	glutathione-S-transferase
	LB	Luria-Bertani Broth
	M	Molar
	ORF	open reading frame
	PCR	polymerase chain reaction
15	rEPF	recombinant Early Pregnancy Factor
	RSP	reverse sequencing primer
	SDS	sodium dodecyl sulphate
	SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
20	Tris	Tris(hydroxymethyl)aminomethane
	USP	universal sequencing primer

**Materials and Methods****Cloning of Human cpn10 Open Reading Frame**

25 Polymerase chain reaction (PCR) was used to initially amplify part of the ORF (274 bp) of the human cpn10 cDNA from a melanoma cell line A2058 cDNA lambda library (Stratagene). A degenerate cpn10 amplimer (P1) was designed from the amino acid sequence VLDDKDYFL corresponding to amino acid residues 83-91 of human cpn10. The primer P1 has the sequence 5' ARRAARTARTCYTTRTCRTC 3' where R is A or G and Y is C
   
 30 or T. The reverse sequencing primer (RSP) was used for PCR amplification (the non-specific primer) as well as for sequencing DNA constructs and has the sequence 5' CAGGAAACAGCTATGAC 3'. The universal sequencing

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primer has the sequence 5' GTAAAACGACGGCCAGT 3'. PCR amplification of the phage library was achieved using a non-specific upstream amplimer (RSP) and P1, each at 0.5  $\mu$ M final concentration, 1.5 mM MgCl<sub>2</sub> (Pharmacia Biotech), 1X polymerase buffer (Boehringer Mannheim) and 5 units of *Thermus aquaticus* DNA polymerase (Boehringer Mannheim) in a final volume of 50  $\mu$ L. For 30 cycles, the parameters were: denaturation at 94°C for 1 min, annealing at 40°C for 30 sec and extension at 72°C for 3 min. A final extension at 72°C for 7 min was followed by a soak cycle at 4°C for 10 min. An aliquot of 1  $\mu$ L was reamplified under the same conditions to increase the copy number.

Two cpn10 specific amplimers encompassing the open reading frame were designed. The upstream primer P2, 5'-GCGCGGATCCATGGCAGGACAAGCGTTTAG-3', was designed from the sequence of the initial PCR fragment. The downstream primer P3, 5'-ATATGAATTCAGTCTACGTACTTTCC-3' was designed from sequence obtained from the Expressed Sequence Tag database via ANGIS (Accession No. HUM00TB037). A 319 bp fragment was amplified from the phage library using the same reaction and cycling conditions as above except the annealing temperature was 50°C.

#### DNA constructs and Analysis

All restriction enzyme digests of PCR products and vectors were performed according to Sambrook *et al.* (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Press, Cold Spring Harbor, NY) using restriction enzymes and their buffers obtained from Boehringer Mannheim. The initial PCR fragment was digested with *Eco* R1 and ligated (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Press, Cold Spring Harbor, NY) into the *Eco* R1 and *Sma* I sites of pBluescript KS(+) (Stratagene) creating the plasmid pRM1 (FIG. 4; partial cpn10 insert 274 bp). The 319 bp product was digested with *Bam* HI and *Eco* R1 and initially cloned into the expression plasmid pGEX-2T (Pharmacia Biotech) creating the plasmid pRM2 (FIG. 5). To confirm its identity, the *Bam* HI-*Eco* R1 fragment was subcloned into pBluescript (SK+)

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(pRM3; FIG. 6) and sequenced. DNA was analysed on 0.8-1.0% (w/v) agarose gels containing ethidium bromide and after electrophoresis was viewed under UV illumination.

Transformation of *E. coli*

5                   Competent *E. coli* DH5 $\alpha$  cells (100  $\mu$ L) were transformed with the plasmids by the heat pulse method (Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press, Cold Spring Harbor, NY). The mixture of cells and DNA (10-100 ng) was placed on ice for 30 min and heat pulsed for exactly 2 min at 42°C and placed back  
10                   on ice for 2 min. The cells were allowed to recover at 37°C with shaking for 1 hr after the addition of 0.9 mL of LB. A 100  $\mu$ L aliquot was plated onto LB agar plates supplemented with Ampicillin at a final concentration of 100  $\mu$ g/mL. After incubation overnight at 37°C, random colonies were selected for further investigation.

15                   DNA sequence determination

                  Restriction fragments of the PCR products were cloned into pBluescript and sequenced in both orientations by the dideoxy chain-termination method using the T7 Polymerase Kit according to the manufacturer's instructions (Pharmacia Biotech). Approximately 2  $\mu$ g of  
20                   plasmid DNA was denatured, ethanol precipitated and annealed to either the USP, RSP or P3. The sequencing reactions were electrophoresed on a 8% acrylamide/46% urea gel. After fixing and drying, X-ray film was exposed to the gel overnight and developed.

Expression and purification of recombinant *cpn10* in *E. coli*

25                   Clones transformed with pRM2 were screened for expression of the Glutathione-S-transferase fusion protein on a small culture scale (2 ml) according to methods described by Smith *et al.* (Smith *et al.*, 1988, Gene 67 (1) 31-40). An overnight culture was diluted, induced to express the fusion protein by the addition of IPTG to 0.1 mM and grown at 37°C for several  
30                   hours. The cells were pelleted, lysed in PBS/0.1% Triton X-100 and the lysate mixed with 50% Glutathione-Agarose beads (Sigma Chemical Company). The recombinant fusion protein was eluted from the affinity beads



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by boiling in SDS loading buffer. An aliquot of the sample was run on a 10% SDS-PAGE gel. The gel was fixed and then stained with Coomassie blue. After confirming the expression of the fusion protein the purification of rcpn10 from the GST moiety was undertaken on a larger scale.

5                Cells were grown and induced as above, the cell pellet resuspended in PBS, sonicated (output level 4, 50% duty cycle, 2 x 30 sec) and the cell lysate stored at -30°C. Lysate from 10 litre cell culture was thawed and rcpn10 isolated by similar techniques to those used by Gearing *et al.* (Gearing *et al.*, 1989, *Biotechnology* 7 1157-1161) for isolation of rLIF. Briefly, Triton X-100 was added to a final concentration of 0.1% and cellular debris removed by centrifugation (15 min, 15000 rpm, 4°C). Ten ml glutathione-Sepharose 4 B gel (Pharmacia - LKB Biotechnology) was added to the supernatant and the slurry mixed for 2 hr, 4°C. The gel was pelleted, washed x 5 with 50 ml PBS/0.1% Triton X-100 once with 50 ml 0.05 M Tris-HCl pH 8.0/0.15 M NaCl and once with 0.05M Tris-HCl pH 8.0/0.15 M NaCl/2.5 mM CaCl<sub>2</sub>. The gel was resuspended in 4 ml of 0.05 M Tris-HCl pH 8.0/0.15 M NaCl/2.5 mM CaCl<sub>2</sub> buffer, 1000 units thrombin (Sigma T6884) added and the slurry was mixed in a shaking waterbath for 1 hr, 37°C. The gel was pelleted, the supernatant retained, and the gel was then washed with 3 x 4 ml 0.05 M Tris-HCl pH 8.0/0.15 M NaCl. These washes and the first supernatant, which contain the rcpn10, were pooled, yielding 4-5 mg recombinant protein. Additional rcpn10, which was non-specifically bound to the gel, was recovered as follows. Four ml 0.05 M Tris-HCl pH 8.0/2 M NaCl was added and the slurry mixed for 2 hr, 4°C.

25                After pelleting, the gel was washed with 3 x 2 ml of this 0.05 M Tris-HCl pH 8.0/2 M NaCl buffer, the washes pooled with the first supernatant, yielding a further approximately 1 mg rcpn10. Protein concentrations were estimated by the method of Lowry *et al.* (Lowry *et al.*, 1951, *J. Biol. Chem.* 193 265-275); proteins were analysed by SDS-PAGE using 15% Tris-Tricine gels (Schagger *et al.*, 1987, *Anal. Biochem.* 166 368-379).

## 2.                APPLICATION OF MAMMALIAN CPN10

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(a) *Assay for cpn10*Antigen

A bacterial fusion protein, GST/cpn10, was expressed and isolated with glutathione-Sepharose, as described for preparation of cpn10.

5 The fusion protein was eluted from the gel by application of 50 mM reduced glutathione in Tris-buffered saline. Eluted fractions were analysed by SDS-PAGE and those containing the most fusion protein were pooled. Protein concentration was determined by the method of Lowry *et al.*, 1951, J. Biol. Chem. 193 265-275.

10 Antibody

Antibodies against the fusion protein were raised in rabbits using an immunisation schedule consisting of 4 x weekly injections followed by at least 4 x monthly boosts. Approximately 10 µg protein, emulsified in Freund's Complete Adjuvant for the first injection and in Incomplete Adjuvant thereafter, was used for each injection. Rabbit serum was screened for anti-  
15 cpn10 antibodies by ELISA using plates coated initially with cpn10 (5 µg/ml) and a streptavidin-biotin detection system (Amersham). The antibody (Ab) titres against cpn10 and against the whole fusion protein (in this case GST/cpn10, 5 µg/ml, was bound to the plate) in serum of rabbit #42 are  
20 shown in FIG. 7. Titration of a serum sample against cpn10, taken from this rabbit after the 4th booster dose, is illustrated in FIG. 8.

Immunoassay

This antibody was then used in a competitive binding assay for detection of cpn10, performed as follows. Anti-serum, diluted 1:32000 and  
25 1:64000 (final dilution; diluent 50 mM sodium phosphate buffer, pH 7.4, containing 0.2% w/v gelatin) was incubated, separately (overnight, 4°C) with various concentrations of cpn10. These mixtures were then tested by ELISA as described above, in plates coated with 5 µg/ml cpn10, as illustrated in FIG. 9. Absorbance values for each antibody/cpn10 mixture are compared with  
30 values obtained for the same antibody dilution incubated without cpn10. The degree of inhibition of binding of antibody to the plate is proportional to the amount of cpn10 in the original antibody-cpn10 mixture; from this, a standard

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curve can be constructed, as shown in FIG. 10.

While #42 is not sensitive enough to detect the very low concentration of cpn10, present in serum, we have established techniques which can:

- 5 (1) produce an anti-cpn10 antibody which displays normal hyperimmunisation properties; thus with known techniques for enhancement of the immune response, an antibody with greater avidity could be produced; and
- (2) produce a response in a standard immunoassay technique.

10 With improved antibodies, application of known methods for enhancement of the detection system and pretreatment of serum to both concentrate and partially purify cpn10 (e.g. by application to C<sub>18</sub> Sep-pak cartridge [Waters] and elution with 80% acetonitrile in Tris-buffered saline, or to immobilised cpn60 in the presence of ATP and elution with EDTA), this  
15 technique, alone or in combination with other immunoassay techniques, could be developed for detection of cpn10 in serum.

Sensitivity of the rosette inhibition test, the EPF bioassay

The rosette inhibition test is non-quantitative and cannot be used to determine the cpn10 concentration in serum with accuracy. The assay may  
20 be used semi-quantitatively by comparing the limiting dose of samples, i.e. the highest dilution of sample giving a positive response in the bioassay. Caution must be exercised with this approach since other substances in complex biological fluids, themselves inactive in the bioassay, can influence the response of active materials.

25 We have determined that the bioassay can detect as little as 5 - 50 ag/ml pure cpn10 (Cavanagh *et al.*, 1994, Eur. J. Biochem. 202 551-560). Based on the observed limiting dose of serum from pregnant women (known inhibitory substances having been removed from early pregnancy serum) and tumour-bearing animals and individuals as well as from rats 24 hr post-partial  
30 hepatectomy, the cpn10 concentration of serum is likely to be in the range 0.1 - 100 pg/ml.

3. TREATMENT WITH CPN10

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(a) *Organ/skin grafts*

*THE EFFECT OF RECOMBINANT CPN10 ON THE SURVIVAL  
OF ALLOGENIC SKIN GRAFTS IN RATS*

5 *Skin grafting*

Skin grafts were exchanged between inbred Lewis and DA rats (~100 g) using the following protocol. Abdominal full thickness skin was sutured onto a similar sized defect created on the lateral thoracic region using standard techniques. A group of six rats were grafted in one session, with each rat receiving one autograft and one allograft. Two Lewis and 2 DA rats received daily x 2 injections of recombinant cpn10 and one Lewis and one DA received buffer, injected around the site of the grafts. Different groups received different doses of cpn10. Injections were continued for 14 days. The grafts were covered with Vaseline gauze, Melolin dressing, plastic wrap and Co-flex elastic bandage. After 7 days, the grafts were examined daily for signs of necrosis. The day of rejection was taken as that on which 50% of the transplanted skin had undergone necrotic degradation.

*Life span of cpn10 activity in serum following injection of recombinant  
cpn10 into mice*

Various doses of recombinant cpn10 (see FIG. 11) were injected *i.p.* into BALB/c mice (~20 g) and the mice bled at various times after administration, commencing at 15 minutes (zero time). Serum was tested for cpn10 activity in the rosette inhibition test (see Morton *et al.*, 1987, Current Topics in Developmental Biology 23 73-92) with spleen cells from C57BL/6 mice. Mice receiving platelet cpn10 were tested in parallel. The half life of cpn10 activity in serum was determined.

*Results*

The results are shown in TABLE 2 and FIG. 11.

There was a significant prolongation of graft survival time following injection of recombinant cpn10 ( $p < 0.001$ , Student's *t* test). The results showed a bell-shaped dose response curve, with the most effective doses being in the range of 2 to 20  $\mu\text{g}$  cpn10 x 2/rat/day. The experiments in

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mice suggest that this recombinant cpn10 has a shorter than expected half life in serum, when compared with platelet cpn10; the half life of 1  $\mu\text{g}$  and 15  $\mu\text{g}$  recombinant cpn10 in serum of mice was only 3 hours and 7 hours respectively, compared with platelet cpn10 (5  $\mu\text{g}$ ), which had a half life of 4 days. However, these results have shown that cpn10 can significantly prolong the viability of allogenic skin grafts in rats. (See TABLE 2).

(b) *Treatment of mammals including humans with cpn10 to promote wound healing*

#### *THE INVOLVEMENT OF CPN10 IN TISSUE REPAIR*

Growth factors are likely to be involved in the healing process, as their initial release from platelets is of fundamental importance in wound repair (Falange, 1993, J. Dermatol. Surg. Oncol. 19 716-720). Platelets have been shown to be a rich source of cpn10 (cpn10; Cavanagh *et al.*, 1994, Eur. J. Biochem 222 551-560) and therefore may be one of the growth factors intimately involved in wound healing. Studies have been carried out to determine the effect of topically-applied recombinant cpn10 (rcpn10) on the healing of full-thickness skin defects created in mice.

#### Methods

Outbred, male Quackenbush mice (aged 8 weeks) were anaesthetized with Nembutal, shaved, skin sterilized with 70% v/v ethyl alcohol and a full thickness defect (8 mm diameter) created in the lateral thoracic region. One  $\mu\text{g}$  rcpn10 in 5  $\mu\text{l}$  Tris-buffered 0.9% w/v sodium chloride (saline) pH 7.4, Tris-buffered saline alone (5  $\mu\text{l}$ ) or saline alone (5  $\mu\text{l}$ ) was applied directly to the wound, which was then covered with Vaseline gauze, Melolin non-adherent dressing and held in place with Co-flex elastic bandage. Twice daily, the mice were lightly anaesthetized with halothane (Fluothane, ICI), the dressings removed, 5  $\mu\text{l}$  of the appropriate solution applied and the wound redressed. At various intervals, i.e. 24 hr, 48 hr, 3 d, 4 d, 5 d, 6 d and 7 d, groups of mice were euthanased with halothane, the wound and surrounding tissue removed and the area of the wound measured.

#### Results

Following treatment with rcpn10, there was a significant

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acceleration of wound contraction, when compared with wounds treated with buffer or saline (FIG. 12). In the wounds treated with cpn10, wound contraction commenced within the first 24 hrs, whereas the control wounds, contraction commenced after 2 days (FIG. 12). From 3 days, there was no significant difference in wound size.

### Conclusions

Cpn10 applied topically to full thickness wounds in mice, accelerates contraction and healing, with the process appearing to commence directly after wounding. Wound contraction in the control mice did not become evident until at least 48 hrs later.

Normally, wound healing takes place in three phases. Phase 1, the inflammatory phase (0-48 hrs), begins immediately after injury and is the time during which activated platelets secrete growth factors into the defect, facilitating fibroblast activation and increasing the activity of cells, e.g. macrophages, involved in the subsequent stages of wound healing. Phase 2, the proliferative phase (2-6 days), begins as the first fibroblasts appear and epidermal cells multiply and migrate to the wound site. Phase 3 is the maturation phase.

Wound contraction does not normally commence in phase 1, also known as the lag phase. During this phase, the shape and size of the excised wound is influenced by elastic forces in the neighbouring skin. These forces increase the initial size of the defect and give it a different shape corresponding to the tension lines present in the skin. As we have shown in the groups of mice treated with buffer or saline, the wounds were enlarged during the first 48 hrs. In contrast, the wounds in mice treated with rcpn10 contracted during this time suggesting that administration of rcpn10 directly to the wound accelerated migration of fibroblasts and deposition of collagen to the wound area. This finding will have enormous significance in the treatment of wounds including burns, as accelerated wound contraction will greatly decrease fluid loss and risk of infection.

(c) *Autoimmune disease*

*THE EFFECT OF CPN10 ON THE DEVELOPMENT OF*

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*EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN RATS,  
AN ANIMAL MODEL OF AUTOIMMUNE DISEASE*

Introduction

Experimental allergic encephalomyelitis (EAE) is an  
5 autoimmune demyelinating disease of the nervous system, induced by  
inoculation of animals with central nervous system myelin basic protein (MBP)  
in adjuvant, and widely studied as an animal model of multiple sclerosis  
(Raine, 1984, Laboratory Investigation 50 608-635). The clinical features of  
EAE in the rat, a commonly studied species, are dramatic overnight weight  
10 loss from day 10 after inoculation, followed by tail weakness and paralysis,  
hindlimb weakness and sometimes paralysis. Forelimb weakness and paralysis  
sometimes occur (Pender, 1986, Journal of Neurological Sciences 75 317-  
328). Experiments were undertaken to determine if administration of rcpn10  
to rats following inoculation, would influence progress of the disease.

15 Methods

EAE model: EAE was induced in inbred female Lewis rats  
(aged ~ 10 weeks) following inoculation with MBP in Freund's adjuvant into  
one footpad. Three groups of rats were included in the study. All were  
inoculated on day 0. Group 1 (n = 4) received no treatment and animals were  
20 not handled during the incubation period of the disease (day 0 to day 8).  
Group 2 (Control group; n = 5) received Tris-buffered saline (0.1 ml) *i.p.* x  
2 daily from day 0 to day 20. Group 3 (Test group; n = 5) received 15 µg  
rcpn10 in Tris-buffered saline (0.1 ml) *i.p.* x 2 daily from day 0 to day 20.  
From day 8, all rats were weighed and examined daily for 30 days.

25 (I) Tail weakness was graded as follows:-

0 = no weakness;

1 = weakness of distal part of the tail only, the distal  
tail failing to curl round the examiner's finger;

2 = weakness of the whole tail but the proximal tail  
30 still being able to be erected vertically against  
gravity;

3 = severe weakness with only a flicker of tail

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movement;

4 = complete flaccid paralysis of the tail.

(II) Hindlimb weakness was graded thus:-

0 = no weakness;

1 = slight dragging of the toes of both hindfeet;

2 = severe dragging of both hindfeet but not of the rest of the hindlimbs;

3 = severe dragging of both hindlimbs, often with both hindlimbs displaced to one side of the body;

4 = total flaccid paralysis of the hindlimbs.

(III) The forelimbs were assessed in a similar way to the hindlimbs.

Total score was the sum of the scores in (I), (II) and (III).

Results

The time of onset of weight loss and period of maximum weight loss in the groups receiving no treatment or receiving injections of buffer alone (Control group) did not differ significantly (Table 3). However, initial weight loss was delayed in the group receiving cpn10 (Test group), compared with the group receiving no treatment, as also was the period of maximum weight loss (Table 3;  $p < 0.001$   $\chi^2$  distribution). There was no significant difference between the means of maximum weight loss in the three groups (Table 3).

Administration of *i.p.* injections 2 x daily to the rats with the necessary handling involved did not affect the time of onset or the severity of the disease but did prolong the course of the disease for several days (day 17 to day 18 as shown in FIG. 13). However, one marked difference between these two groups was the recurrence of severe disease in the Control group at day 22, persisting until day 30. In the group receiving no treatment, mild disease only recurred in 3 rats on days 27 and 28.

As with early weight loss, weakness and paralysis of the tail and limbs were delayed in the Test group, receiving rcpn10, compared with the Control group (FIG. 14: day 12,  $p < 0.01$ ; day 13,  $p < 0.05$ , Heteroscedastic *t* test). In the Test group over the period from 14 to 16 days,



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only one rat developed severe disease, similar to that developed by the Control rats. The remaining 4 rats only developed mild disease during this period (FIG. 15,  $p < 0.95$ , Heteroscedastic  $t$  test). Severe disease did not recur in the Test group, during the examination period; one rat developed mild disease at day 22 and the remaining rats from day 27 to day 30.

#### Conclusions

Treatment itself, i.e. administering fluid *i.p.* x 2 daily to rats, did not effect the onset or severity of the disease but did marginally extend its time course. Furthermore, severe disease did recur during the observation period in the rats receiving daily injections of Tris-buffered saline but not in the rats receiving no treatment.

The most interesting observation made in the this study was that treatment of the rats with cpn10 did significantly delay the onset and modify the clinical features of the disease in 4 out of 5 rats. It also prevented the recurrence of severe disease during the time these animals were under observation.

#### (d) *Infertility and miscarriage*

A further aspect of the invention is the treatment of fertility and/or miscarriages with the administration of cpn10. This is of importance where the problem arises from the lack of cpn10. Experimental support below demonstrates the requirement for cpn10 during embryo development.

To create the situation of reduced cpn10 concentration, anti-cpn10 antibodies were developed and used. There is no animal model system available. It follows therefore from the experimental support that administration of cpn10 to increase the concentration of cpn10 during pregnancy will overcome the aforementioned problems of infertility and miscarriage.

#### *Synthesis of cpn10 derived peptides*

Peptides were synthesized to correspond with an N-terminal fragment (N-peptide i.e. Ac-AGQAFRKFLPLC) and an internal fragment (I-peptide i.e. EKSQGKVLQATC) of cpn10.

#### *Conjugation of peptides to ovalbumin*

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Peptides were conjugated to ovalbumin by the hetero-bifunctional reagent SPDP, following manufacturer's instructions (Pharmacia-LKB Biotechnolokogy, Uppsala, Sweden).

#### *Immunisation Schedules*

5 Adult outbred New Zealand rabbits were immunised with one of the conjugates in 4 x weekly injections followed by several monthly boosts.

For injection, the antigen was dialysed into 0.9% saline (Mr 12-15000 cut off dialysis tubing, Visking, Union Carbide, IL, USA) and emulsified with an equal volume of Freund's adjuvant (complete for the first  
10 injection, incomplete thereafter). Immunisations were via the s.c. route.

#### *Screening of anti-serum*

Antisera were tested in an ELISA against the relevant antigens (viz. I-peptide or N-peptide; ovalbumin) (5 mg/ml). Bound IgG was detected by the biotin-streptavidin system (Amersham) with o-phenylene diamine as  
15 substrate. Absorbance was read at 492 nm.

IgG was precipitated from anti-serum by 45% ammonium sulphate and the concentration determined by Lowry and gel electrophoresis. The IgG preparations were tested in an ELISA (Table 4) against the immunising peptide, conjugated to bovine serum albumin. The preparations  
20 were also tested for their ability to neutralise activity of mouse pregnancy serum in the rosette inhibition test. Various concentrations of antibody were incubated with an equal volume of serum, then the mixtures tested for activity in the rosette inhibition test. The lowest concentration of antibody that could completely neutralise activity was determined (see Cavanagh *et al.*, 1994, Eur.  
25 J. Biochem. 222 551-560). Ten pg of anti-N-peptide Ab neutralised the activity of 1 ml pregnancy serum while 4 ng anti-I-peptide Ab was needed for complete neutralisation.

#### *Passive Immunisation*

30 Mature outbred male and female Quackenbush mice were caged in pairs at 7.30 a.m. and separated at 8.30 a.m. Female mice with vaginal plugs were injected with anti-N-peptide/ovalbumin, anti-I-peptide/ovalbumin or anti-ovalbumin IgG preparations at 9.00 a.m. and 5.00 p.m. on days 1 (day of

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5 mating) and 2 of pregnancy. The dose of specific IgG injected in the 2 dose regimen was estimated as approximately 1 mg/mouse/day. On day 7, mice were euthanased with CO<sub>2</sub>, uteri examined for implanted embryos and the number of corpora lutea (CL) counted. In each group, the number of embryos/CL in the mice treated with the test IgG was compared with the number receiving the same dose of control IgG ( $\chi^2$  test).

### Results

10 The results, shown in Table 5 clearly demonstrate that neutralisation of cpn10 in pregnancy serum can adversely affect embryonic viability in the early stages of pregnancy. The ability of antibodies to neutralise activity in the rosette inhibition test is an *in vitro* monitor of their ability *in vivo* to adversely affect pregnancy.

### OTHER ASPECTS OF THE INVENTION

15 In another aspect of the invention, further work has now elucidated two regions of the molecule with biological activity, corresponding with residues 1-11 and 34-44 in rat and human cpn10.

The invention therefore includes within its scope amino acid sequences:-

- 20 (i) AGQAFRKFLPL;
- (ii) Ac-AGQAFRKFLPL where Ac is acetyl;
- (iii) EKSQGKVLQAT

which may function as active centres of the cpn 10 molecule.

The invention also includes within its scope molecules (i), (ii) and (iii) having one or more end sequences A<sub>1</sub> and A<sub>2</sub> ie.

- 25 (iv) A<sub>1</sub>AGQAFRKFLPLA<sub>2</sub>;
- (v) AGQAFRKFLPLA<sub>2</sub>;
- (vi) A<sub>1</sub>AGQAFRKFLPL;
- (vii) Ac-A<sub>1</sub>AGQAFRKFLPLA<sub>2</sub>;
- (viii) Ac-AGQAFRKFLPLA<sub>2</sub>;
- 30 (ix) Ac-A<sub>1</sub>AGQAFRKFLPL;
- (x) A<sub>1</sub>EKSQGKVLQATA<sub>2</sub>;
- (xi) EKSQGKVLQATA<sub>2</sub>;

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(xii) A<sub>1</sub>EKSQGKVLQAT;

wherein A<sub>1</sub> and A<sub>2</sub> are amino acid sequences which may be added to one or each end of molecules (i) through (xii) and wherein Ac is acetyl.

5 In the above molecules (i) through (xii), it will be appreciated such molecules also include within their scope a single amino acid addition, deletion or substitution.

10 In regard to the use of cpn10 in regard to treatment of autoimmune disease, relevant diseases that may be treated by administration of cpn10 include insulin dependent diabetes mellitus, rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, Graves disease and multiple sclerosis. This is evident from the relevant supporting data in regard to the EAE rat model provided herein.

15 In relation to the use of cpn10 in relation to treatment of organ transplants, skin grafts, the relevant supporting data given herein refers to the rat skin graft model described herein.

In relation to the use of cpn10 in relation to infertility treatment or prevention of miscarriage, the relevant supporting data refer to the effect of cpn10 antibody on embryonic development and implantation in mice.

20 In relation to the use of cpn10 in relation to wound healing and tissue repair or regeneration of tissue, this means that cpn10 can be used in treatment of burns, surgery, trauma, skin ulcers including bed sore and diabetic ulcers, infectious diseases involving tissue and organ damage (e.g. hepatitis), metabolic disease involving tissues and organ damage (e.g. liver cirrhosis) and degenerative disease involving tissue or organ damage. The support for these conclusions is given in the mouse wound model referred to herein and the liver regeneration data after partial hepatectomy in rats discussed in Quinn *et al.*, 1994, Hepatology 20 in press.

25 The data referred to herein also provides clear support for the use of cpn10 in treatment of inflammatory conditions including inflammatory bowel disease and infectious disease. Such data as described herein includes references drawn from the immunosuppressive effect of cpn10 in the rat EAE and skin graft models. This is also supported by Rolfe *et al.*, 1983, Clin. exp.

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Immunol. 51 45-52 and Nature 278 No. 5705 649-651 showing that EPF can reduce delayed type hypersensitivity in mice.

5 The use of cpn10 in treatment of allergic disease including allergic rhinitis, asthma, atopic dermatitis, acute urticaria and drug hypersensitivity is also fully supported by the immunosuppressive effect of cpn10 in the rat EAE and skin graft models. This conclusion can also be drawn from Rolfe *et al.*, 1983, Clin. exp. Immunol. 51 45-52 and Noonan *et al.*, 1979, Nature 278 No. 5705 649-651 showing effect of EPF in reducing delayed type hypersensitivity in mice.

10 The use of cpn10 in relation to diagnosis of tumours and/or monitoring patients after surgical removal of tumours is supported by the reference Quinn *et al.*, 1992, Cancer Immunol. Immunother. 34 265-271.

15 In regard to dosages that may be employed concerning administration of cpn10, a convenient dosage would be of the order of 1-1000  $\mu\text{g/kg}$  of body weight and more preferably 50-200  $\mu\text{g/kg}$  of body weight.

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TABLE 1

Sample	Limiting Dose (log reciprocal)	
	Untreated	+ 5/341
Human platelet EPF (50 $\mu$ g/ml)	13	< 2
Rat liver cpn10 (50 $\mu$ g/ml)	13	< 2
<i>E. coli</i> cpn10 ( <i>groES</i> ) (50 $\mu$ g/ml)	NA	NT

TABLE 2

TREATMENT	SKIN GRAFT SURVIVAL TIME	
rEPF/cpn10 (dose x 2/rat/day)	Lewis $\rightarrow$ DA Days $\pm$ SD (n)	DA $\rightarrow$ Lewis Days $\pm$ SD (N)
buffer alone	8.7 $\pm$ 0.75 (7)	9.1 $\pm$ 0.83 (8)
1 $\mu$ g	-	9.0 $\pm$ 1.0 (3) (NS)
5 $\mu$ g	14.0 $\pm$ 1.6 (4)*	14.5 (2)
20 $\mu$ g	15.2 $\pm$ 0.92 (4)*	12.5 (2)
70 $\mu$ g	10.0 (2)	11.0 (2)

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TABLE 3

Treatment Group	n	Onset of weight loss (day)	Max. period of weight loss (day)	p'	Max. weight loss (% wt. at d 10)	p'
No treatment	4	11	14-16		15.4	
Buffer (Control)	5	11	15-17	NS	13.9	NS
cpn10 (Test)	5	12-14	17-19	p < 0.001	13.9	NS

TABLE 4

Antibodies (mg/ml)	Titre (reciprocal serum dilution)	
	N-peptide (5 µg/ml)	I-peptide (5 µg/ml)
Anti-N-peptide	128000	< 1000*
Anti-I-peptide	< 1000*	32000
Anti-ovalbumin	< 1000*	< 1000*

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TABLE 5

Antibody (total dose 2 mg/mouse)	No. of animals in group	Corpora lutea/mouse (mean $\pm$ sem)	Embryo/mouse (mean $\pm$ sem)	p*
Anti-N- peptide- ovalbumin	6	19.1 $\pm$ 1.2	10.6 $\pm$ 3.8	< 0.05
Anti-I- peptide- ovalbumin	6	20.8 $\pm$ 0.8	17.1 $\pm$ 1.1	< 0.02
Anti- ovalbumin	5	17.8 $\pm$ 1.0	16.8 $\pm$ 0.5	NS



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**TABLE LEGENDS****TABLE 1**

Activity of cpn 10 in the rosette inhibition test

**TABLE 2**

Skin graft survival time.

p value when compared with control group receiving buffer alone

\* p &lt; 0.001

**TABLE 3**

Time of Initial Weight Loss and Maximum Weight Loss in the Three Treatment Groups during the Course of EAE.

\*  $\chi^2$  distribution\* Student's *t* test**TABLE 4**

Anti-N-peptide, anti-I-peptide and control anti-ovalbumin antibodies tested in an ELISA against N-peptide and I-peptide.

\* 1 in 1000 was the lowest dilution tested.

**TABLE 5**Effect of passive immunization of confirmed-mated mice at days 1 and 2 *p.c.*, with antibodies to cpn10-derived peptides, on the number of of implanted embryos and corpora lutea present at day 7 *p.c.*\* (Heteroscedastic *t*-test).

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**FIGURE LEGENDS****FIG. 1a**

Purification of EPF. Heat extracted human platelets (100 units) were fractionated on SP-Sephadex and Heparin Sepharose, then applied to a TSK-Phenyl 5PW column and eluted with a reverse salt gradient. Fractions were tested in the rosette inhibition test (based on EPF's capacity to augment the rosette inhibiting activity of an immunosuppressive antilymphocyte serum).

**FIG. 1b**

Active fractions (n) from (a) were fractionated by RP-HPLC-1.

**FIG. 1c**

Active fractions (n) from (b) were fractionated by RP-HPLC-2.

**FIG. 1d**

Active fractions (n) from (c) were fractionated by RP-HPLC-3.

**FIG. 1e**

Interaction of immobilised monoclonal anti-EPF antibody 5/341 with active fractions from (d) and equivalent fractions from human pregnancy serum, 6 d gestation (10 ml); human pregnancy urine, up to 1 month gestation (10 litre); medium conditioned by oestrous mouse ovaries (100) stimulated with prolactin and mouse embryo-conditioned medium (ovary CM); serum free medium conditioned by the bovine kidney cell line MDBK (MDBK-CM; ATCC CCL 22, 10 litre); rat serum obtained 24 h post-partial hepatectomy (post-pH, 10 ml); rat liver obtained 24 h post-pH (40 g); all fractionated as in (a) to (d). Anti-EPF bound and unbound fractions were tested in the rosette inhibition test, specificity was demonstrated by comparison with a parallel experiment using irrelevant antibody in which activity was not bound.

**FIG. 2a**

Analysis of EPF purified from 300 units human platelets as in FIG. 1A. Determination of monomeric size. Iodinated EPF was fractionated by SDS-PAGE,<sup>29</sup> the gel sliced (2 mm wide slices) and the distribution of radioactivity and biological activity compared. (Inset) Direct Coomassie Blue staining of the same preparation.

**FIG. 2b**

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Ion-spray mass spectrum of EPF, displayed as multiply protonated molecular ions. (Inset) Computer reconstruction as molecular mass.

**FIG. 2c**

Amino acid sequence (single letter code) of peptides derived from human EPF, compared with rat cpn10 (underlined). EPF was digested with endoproteinase lys C and endoproteinase glu C, the resultant peptides separated by RP-HPLC and sequenced. The sequence of individual fragments is shown; all except 74-101 were derived from the lys digest.

**FIG. 3**

Interaction of EPF and cpn60 (*groEL*).

**FIG. 3a**

Peak fractions in the excluded volume of a TSK G3000SW gel permeation column, following application of a cpn60-EPF mixture +Mg<sup>2+</sup>ATP, were analysed by SDS-PAGE (Schagger *et al.*, 1987) and stained with silver (Morrissey, 1981). Left lane, +ATP; right lane -ATP. (Cpn60 is a decatetramer, M, 840 000; column exclusion limit > 300 000. Higher M<sub>r</sub> bands on SDS gel are oligomeric forms of *groEL*).

**FIG. 3b**

Immobilised cpn60 was mixed with human pregnancy serum (6 d gestation) in the presence or absence of Mg<sup>2+</sup>ATP. Unbound and bound fractions (the latter recovered from the gel by removal of ATP with EDTA) were then tested in the rosette inhibition test. Results are expressed as limiting dose, the highest dilution of sample giving a positive result in the rosette inhibition test.

**FIG. 4**

pRM1

**FIG. 5**

pRM2

**FIG. 6**

pRM3

**FIG. 7**

Preparation of antibodies to cpn10

**FIG. 8**

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Detection of anti-cpn10 antibodies in rabbit serum by ELISA

**FIG. 9**

Competitive binding assay for cpn10

**FIG. 10**

% inhibition of antibody binding

**FIG. 11**

Time course of recombinant cpn10 and platelet cpn10 activity in serum of mice after injection *i.p.*

**FIG. 12**

The effect of rcpn10 on wound contraction in mice. Wounds ( $\sim 45 \text{ mm}^2$ ) were created in mice,  $1 \mu\text{g}$  rcpn10 or control solutions ( $5 \mu\text{l}$ ) applied topically x 2 daily and the size of wound area on groups of mice measured at times indicated (means  $\pm$  SD); d 0, n = 10, d 1 - d 3, n = 3, d 4 - d 7, n = 2 \* p < 0.05 compared with buffer control group.

**FIG. 13**

Effect of the treatment regimen on development of EAE in rats.

**FIG. 14**

Effect of cpn10 on development of EAE in rats.

**FIG. 15**

Effect of cpn10 on development of EAE in rats.

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CLAIMS

1. A process for the detection of cpn10 in serum or other biological fluids including the steps of:-
  - (i) raising antibody to cpn10;
  - (ii) reacting said antibody with a sample of biological fluid suspected of containing cpn10; and
  - (iii) detecting the presence of cpn10 in said sample by a signal amplification resulting from production of a cpn10-antibody complex.
2. A process as claimed in Claim 1 wherein the antibody is raised against a fusion protein GST/cpn10 or peptides derived from cpn10.
3. A process for the detection of cpn10 in serum or other biological fluids using a rosette inhibition assay including the step of reacting a sample of biological fluid suspected of containing cpn10 with lymphocytes, anti-lymphocyte serum and complement in the presence of heterologous erythrocytes and determining the presence of cpn10 by quantifying the amount of anti-lymphocyte serum to inhibit rosette formation.
4. A process for promotion of cell growth or immunosuppression including the step of administration of cpn10 to a mammalian subject.
5. A process as claimed in Claim 4 for enhancing wound healing tissue repair or tissue regeneration including the administration of cpn10 to a subject suffering from wounds or tissue damage.
6. A process as claimed in Claim 4 for treatment of autoimmune disease including the step of administration of cpn10 to a subject suffering from autoimmune disease.
7. A process as claimed in Claim 4 for enhancing organ or skin graft viability including the step of administration of cpn10 to a subject to which said grafts have been applied.
8. A process as claimed in Claim 4 for use in treatment of inflammatory conditions including the step of administration of cpn10 to a subject.
9. A process as claimed in Claim 4 for use in treatment of allergic

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disease including the step of administration of cpn10 to a subject.

10. A process as claimed in Claim 4 for use in prevention of infertility or prevention of miscarriage including the step of administration of cpn10 to a subject.
11. Recombinant cpn10 having the sequence  
GSAGQAFRKFLPLFDRVLVERSAAETVTKGGIMLPEKSQGVQLQATVEA  
VGSGSKGKGGEIQVSVKEGDKVLLPEYGGTKVVLDDKDYFLFRDGDIL  
GKYVD.
12. A peptide having an amino acid sequence AGQAFRKFLPL.
13. A peptide having an amino acid sequence Ac-AGQAFRKFLPL including a single amino acid deletion, addition or substitution.
14. A peptide having an amino acid sequence EKSQGVQLQAT including a single amino acid deletion, addition or substitution.
15. A peptide having an amino acid sequence A<sub>1</sub>AGQAFRKFLPLA<sub>2</sub> including a single amino acid deletion, addition or substitution and wherein A<sub>1</sub> and A<sub>2</sub> are amino acid sequences.
16. A peptide having an amino acid sequence AGQAFRKFLPLA<sub>2</sub> including a single amino acid deletion, addition or substitution and wherein A<sub>2</sub> is an amino acid sequence.
17. A peptide having an amino acid sequence A<sub>1</sub>AGQAFRKFLPL including a single amino acid deletion, addition or substitution and wherein A<sub>1</sub> is an amino acid sequence.
18. A peptide having an amino acid sequence Ac-A<sub>1</sub>AGQAFRKFLPLA<sub>2</sub> including a single amino acid deletion, addition or substitution and wherein A<sub>1</sub> and A<sub>2</sub> are amino acid sequences.
19. A peptide having an amino acid sequence Ac-AGQAFRKFLPLA<sub>2</sub> including a single amino acid deletion, addition or substitution and wherein A<sub>2</sub> is an amino acid sequence.
20. A peptide having an amino acid sequence Ac-1<sub>1</sub>AGQAFRKFLPL including a single amino acid deletion, addition or substitution and wherein A<sub>1</sub> is an amino acid sequence.
21. A peptide having an amino acid sequence

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$A_1$ EKSQGKVLQATA $_2$  including a single amino acid deletion, addition or substitution and wherein  $A_1$  and  $A_2$  are amino acid sequences.

22. A peptide having an amino acid sequence EKSQGKVLQATA $_2$  including a single amino acid deletion, addition or substitution and wherein  $A_2$  is an amino acid sequence.

23. A peptide having an amino acid sequence  $A_1$ EKSQGKVLQAT including a single amino acid deletion, addition or substitution and wherein  $A_1$  is an amino acid sequence.

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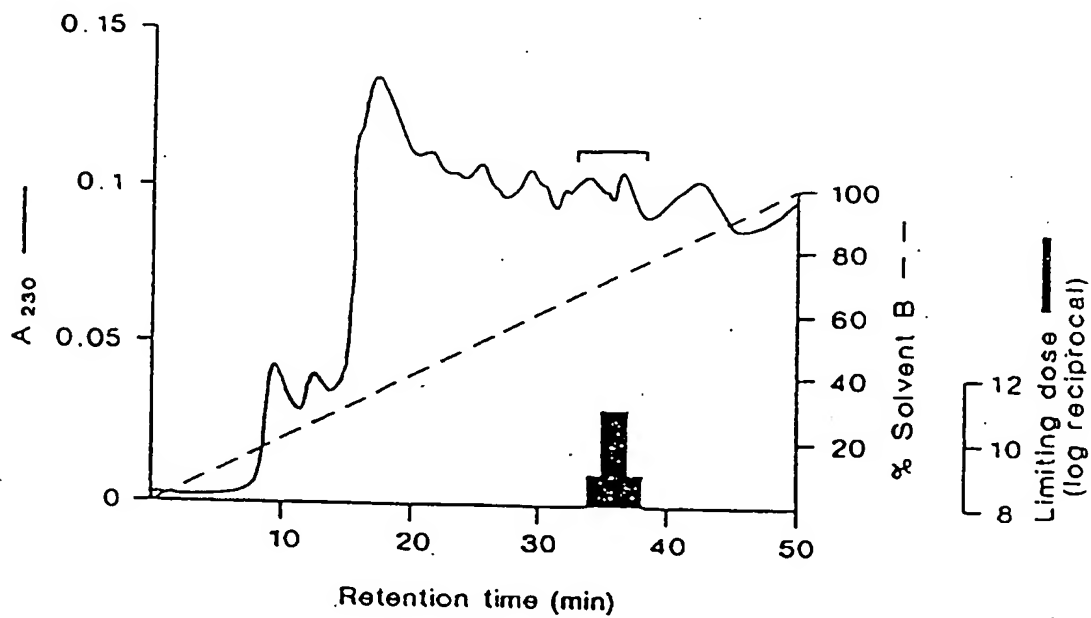


FIG 1a

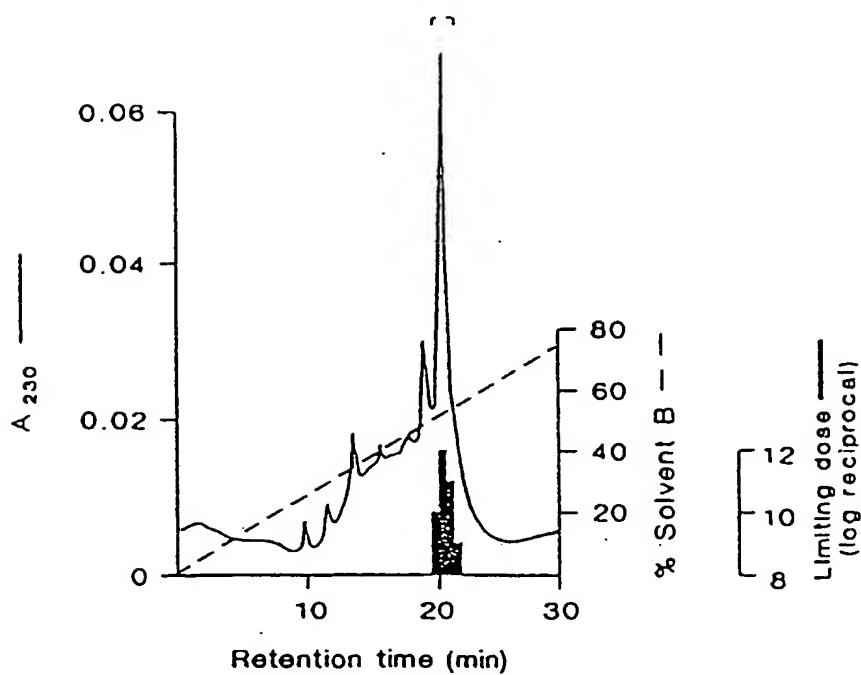


FIG 1b

SUBSTITUTE SHEET (Rule 26)



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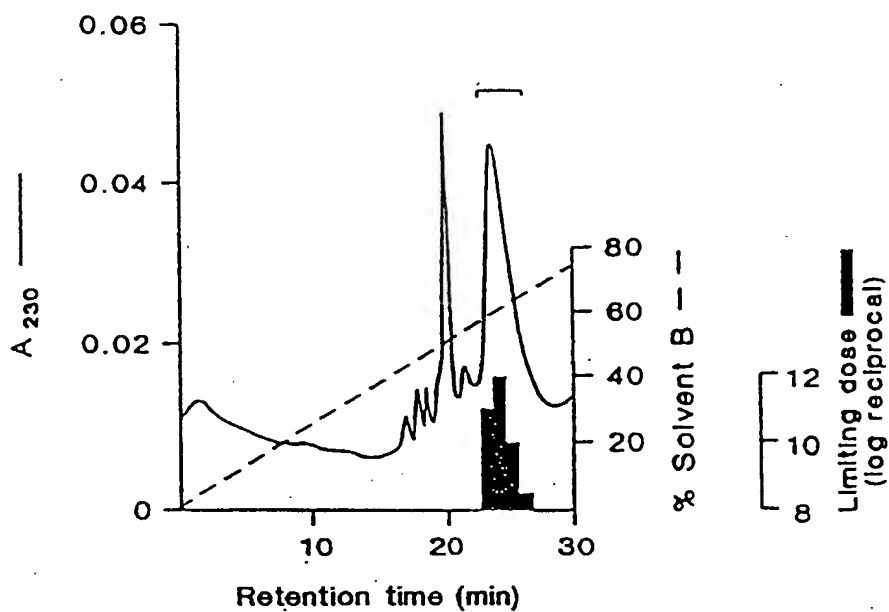


FIG 1c

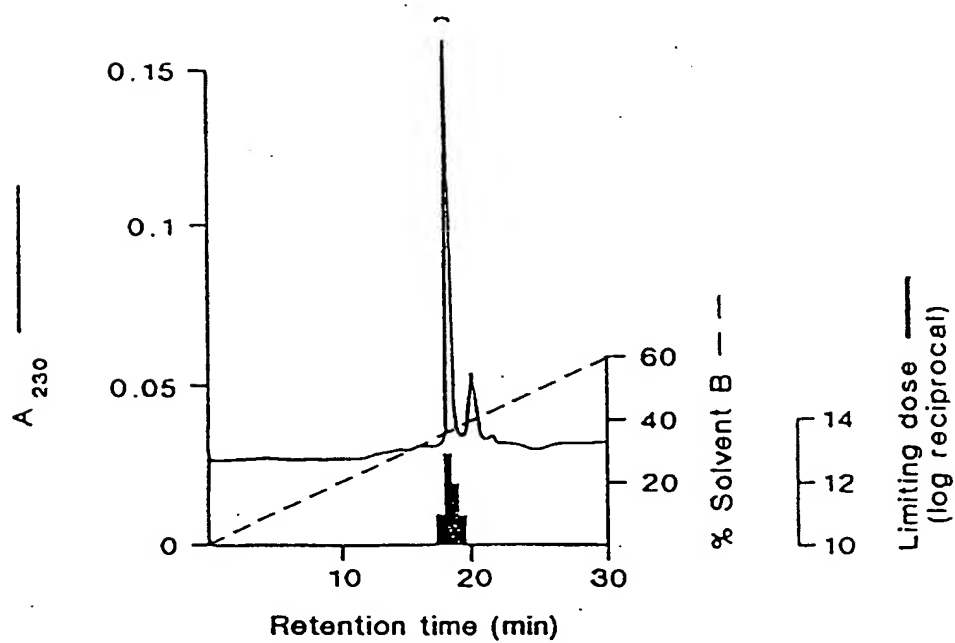


FIG 1d

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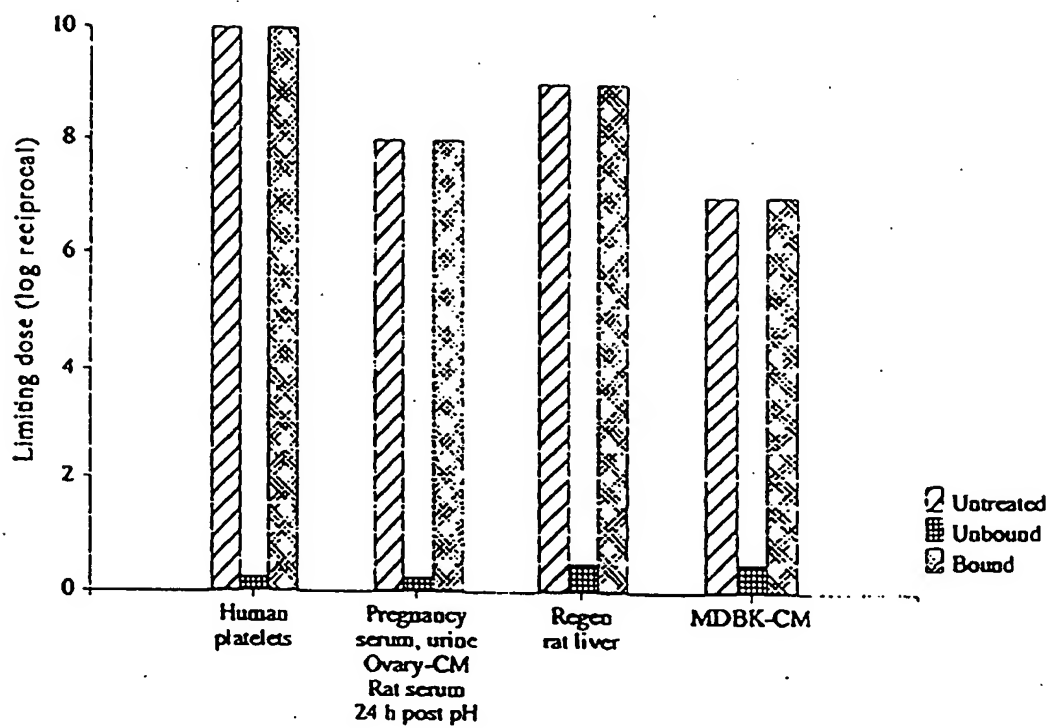


FIG 1e

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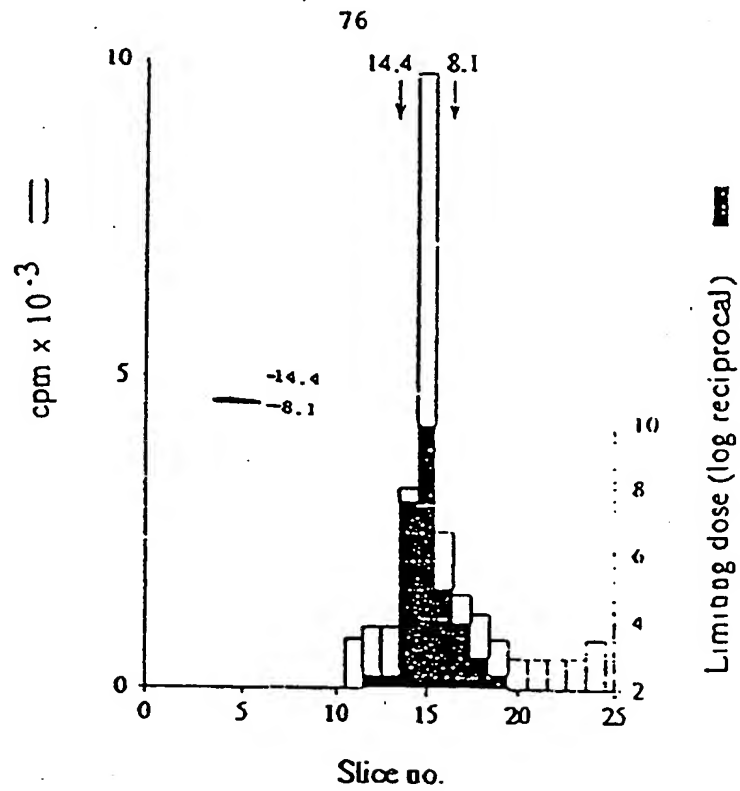


FIG 2a

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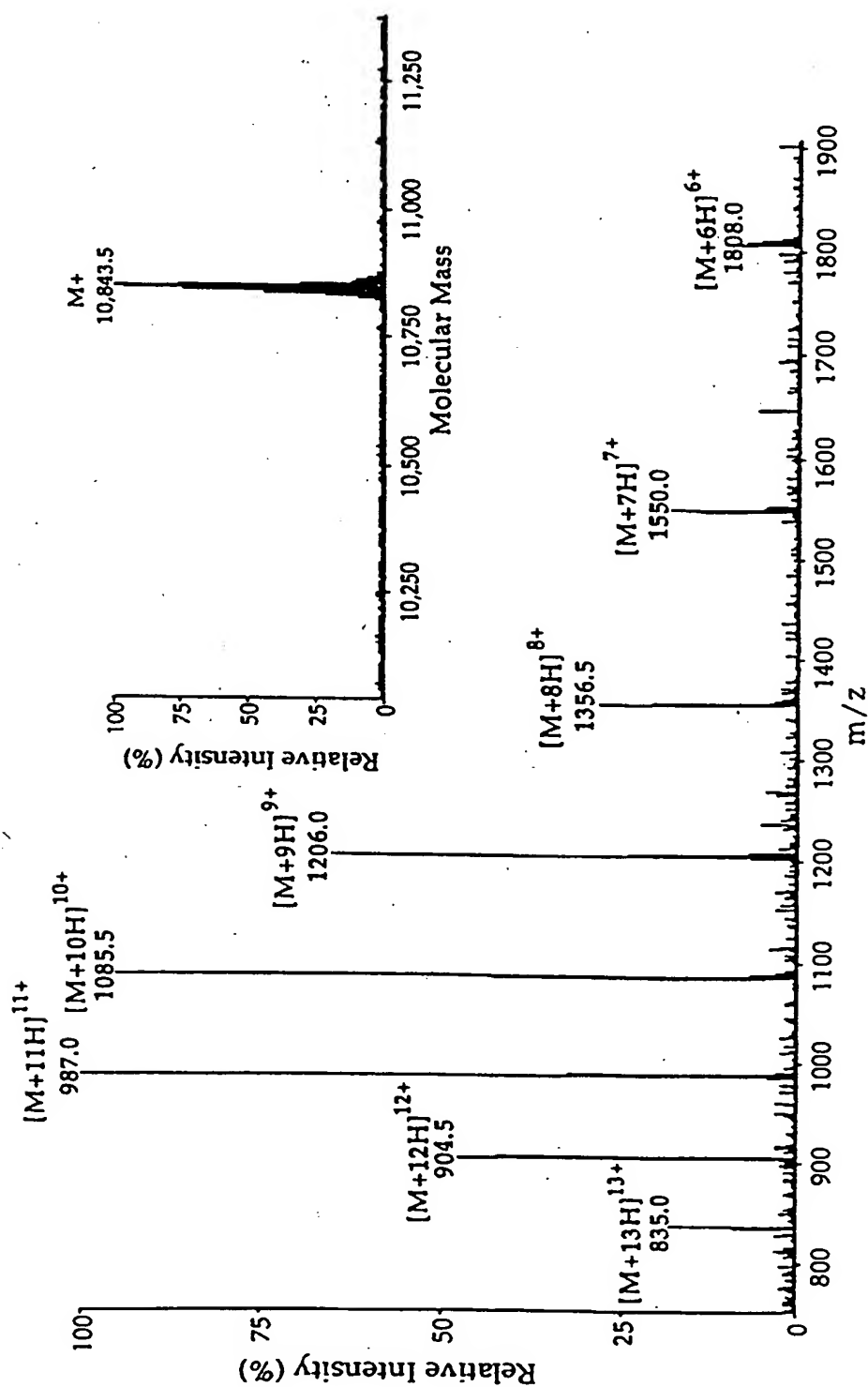


FIG 2b

SUBSTITUTE SHEET (Rule 26)

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Human EPF )											KV	LXATVAVGS
											KSQKV	LQATVAVGX
											MLPEKXQG	
Rat cpn10											MLPEKSQKV	LQATVAVGS
Human EPF )												
Rat cpn10												

FIG 2c

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EPF —

+ATP -ATP

FIG 3a

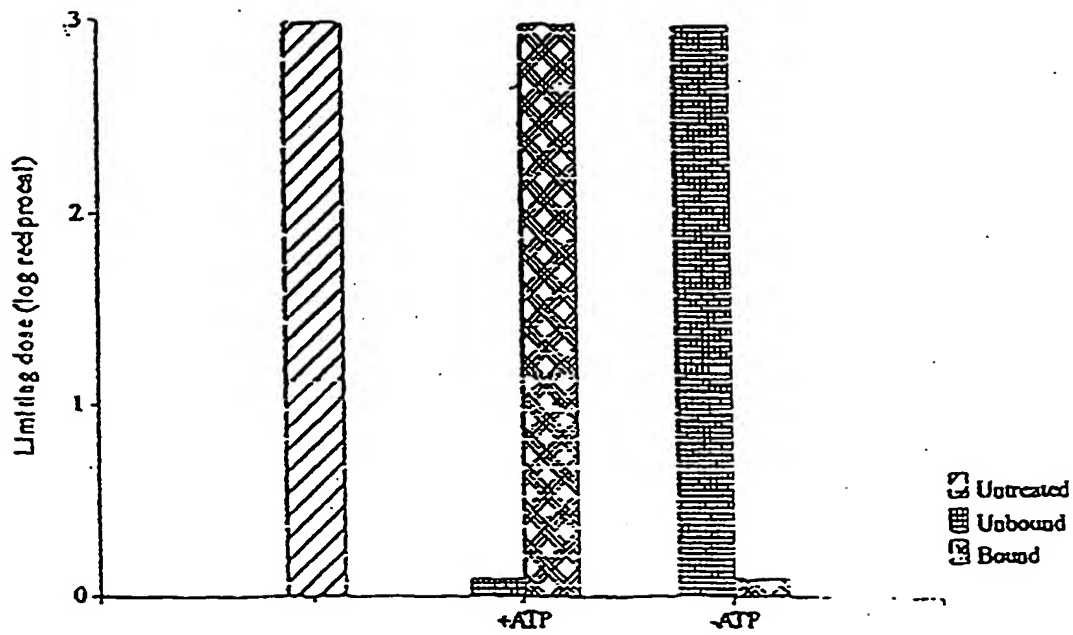


FIG 3b

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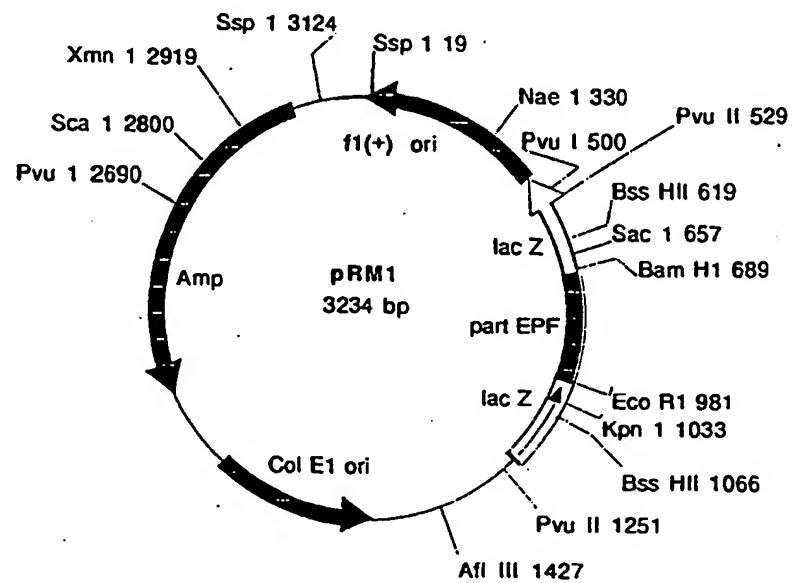


FIG 4

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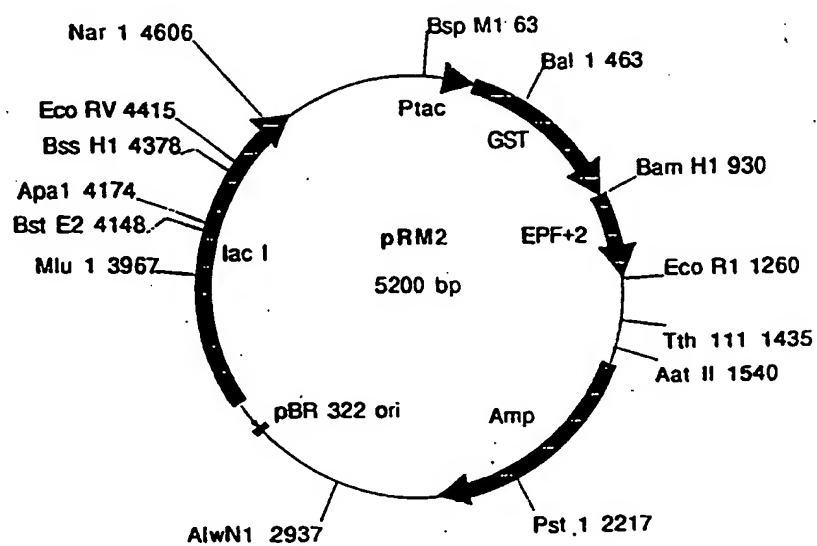


FIG 5



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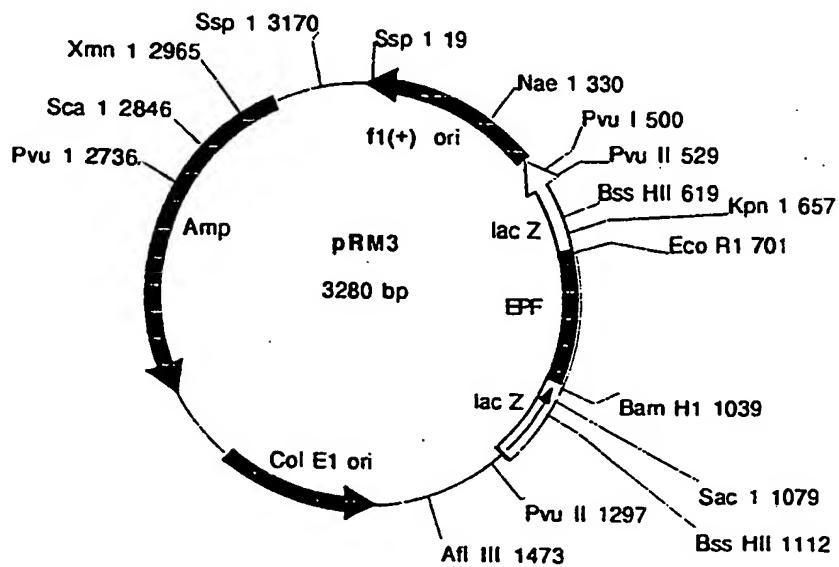


FIG 6

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Fusion protein (GST:rcpn10)

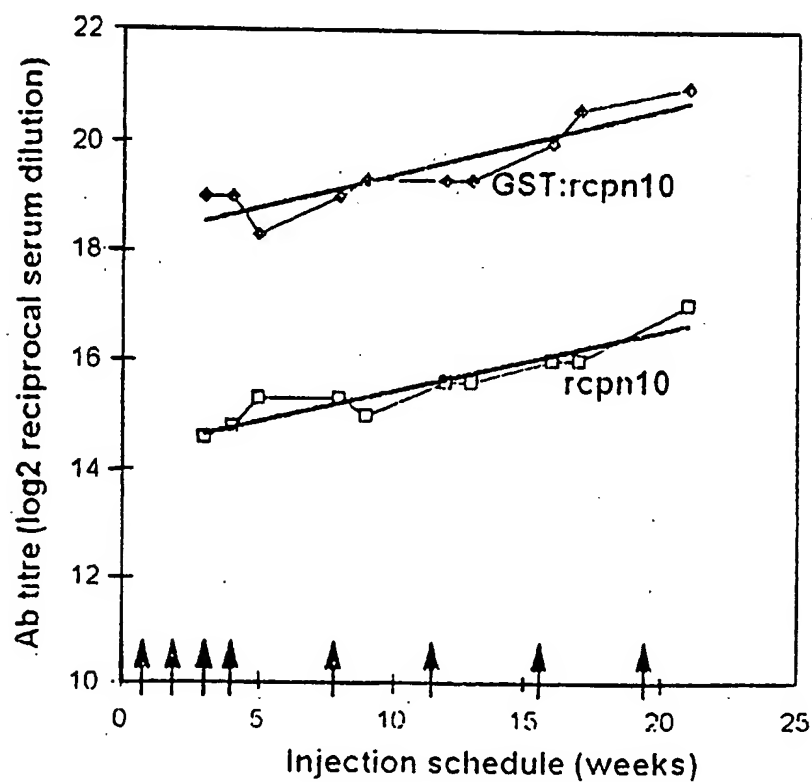
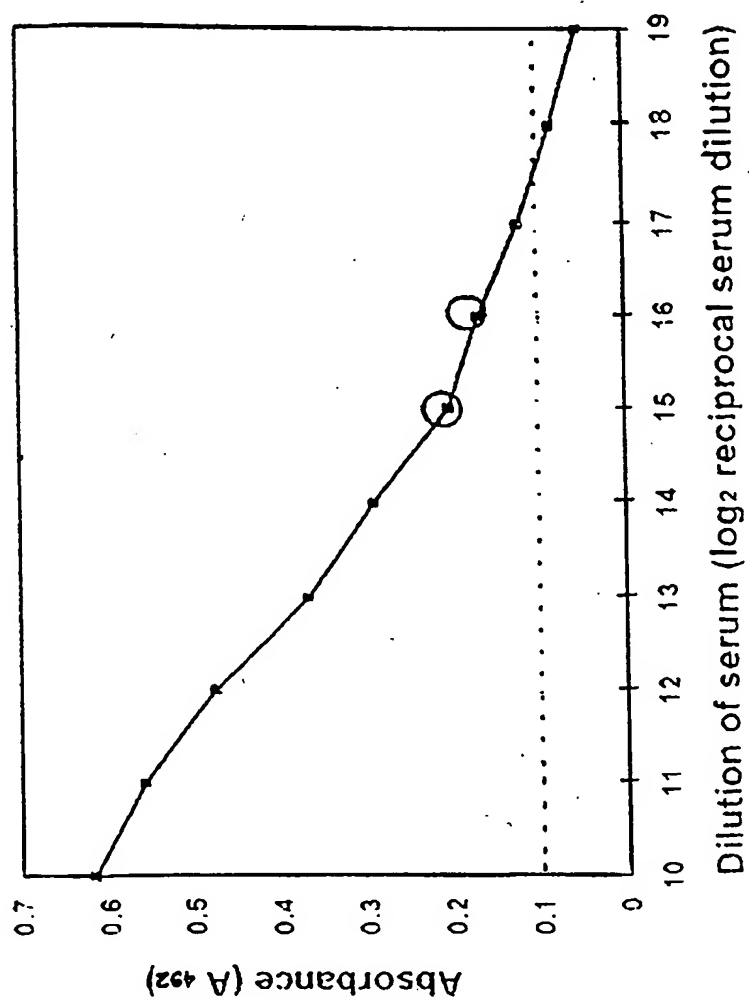


FIG 7

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O = dilutions used in competitive binding assay

FIG 8

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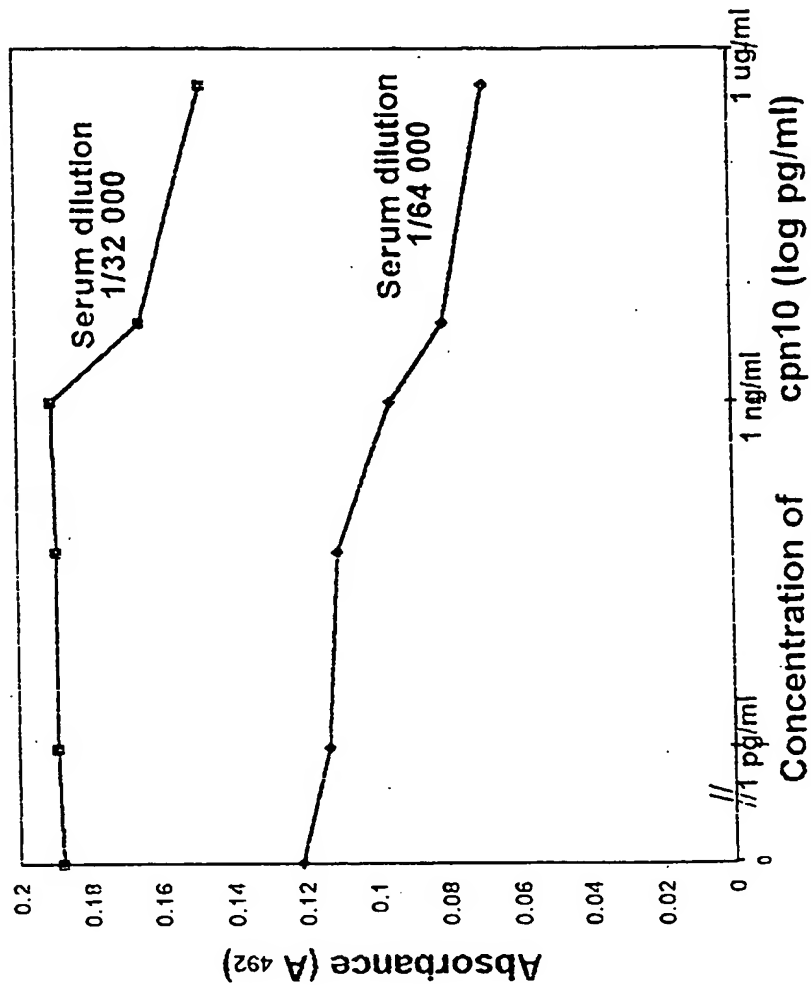
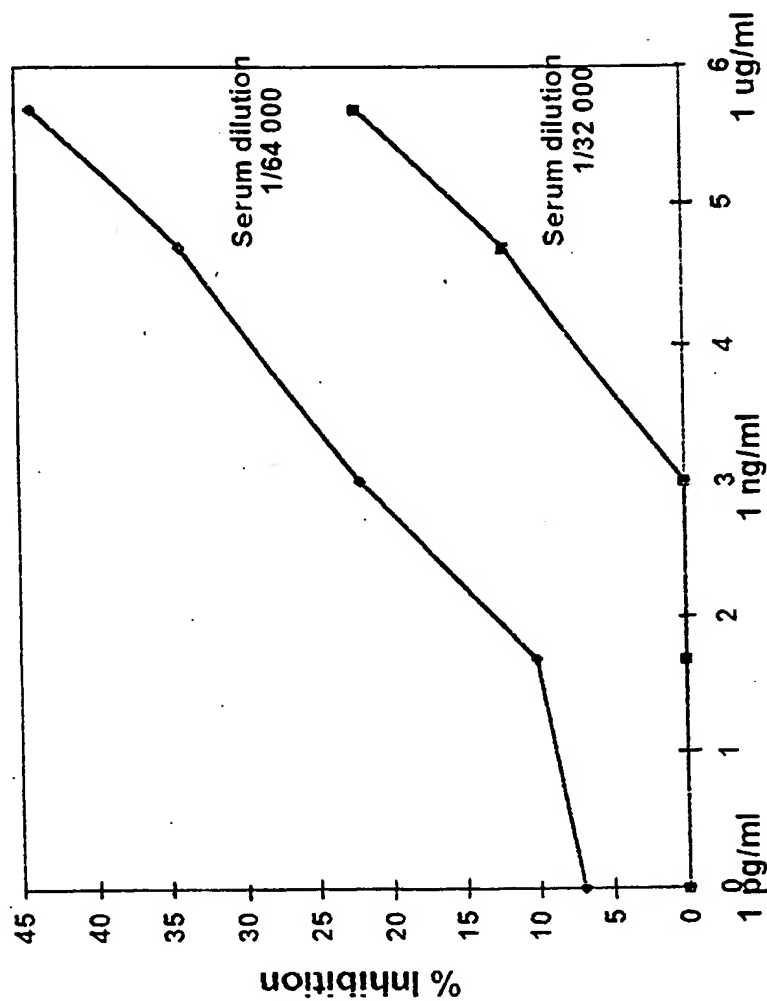


FIG 9

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Concentration of cpn10 (log pg/ml)

FIG 10

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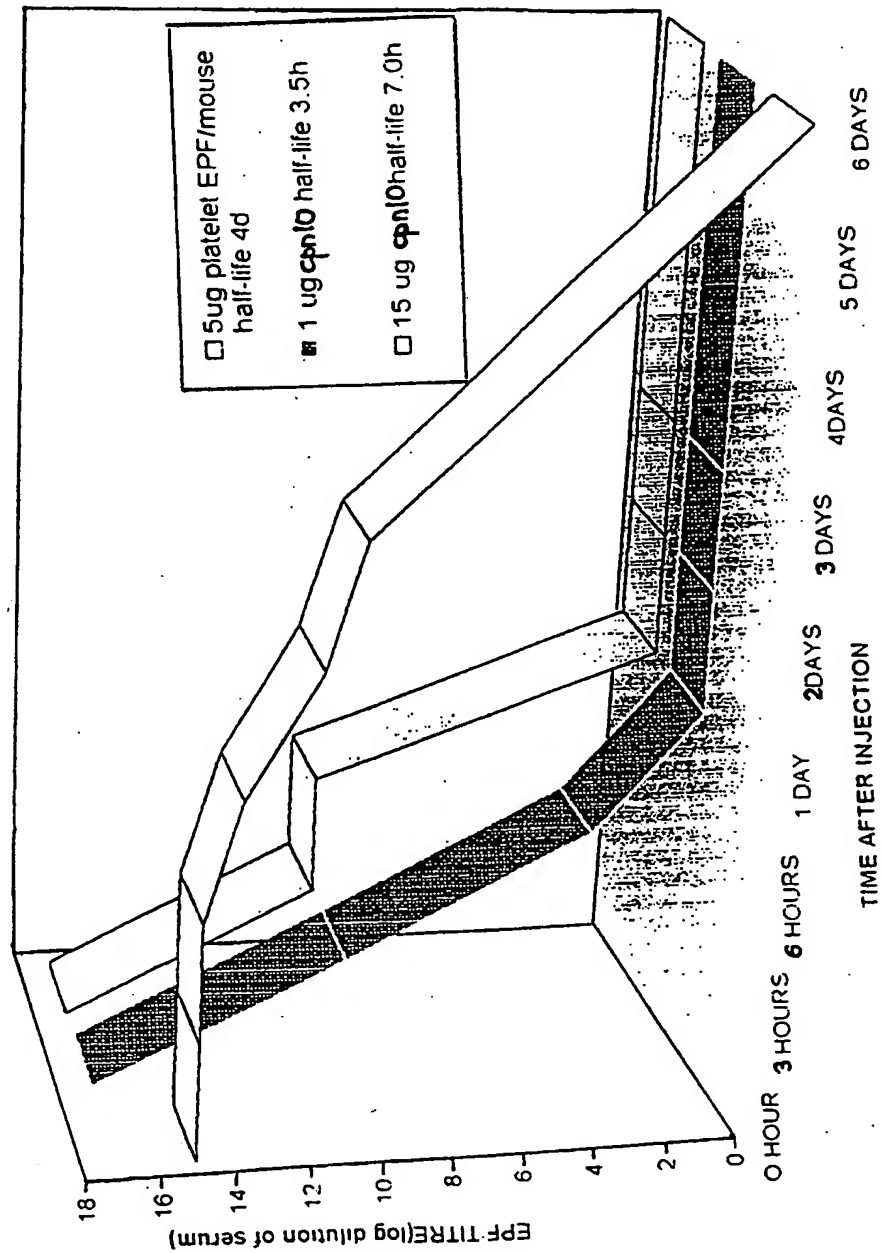


FIG 11

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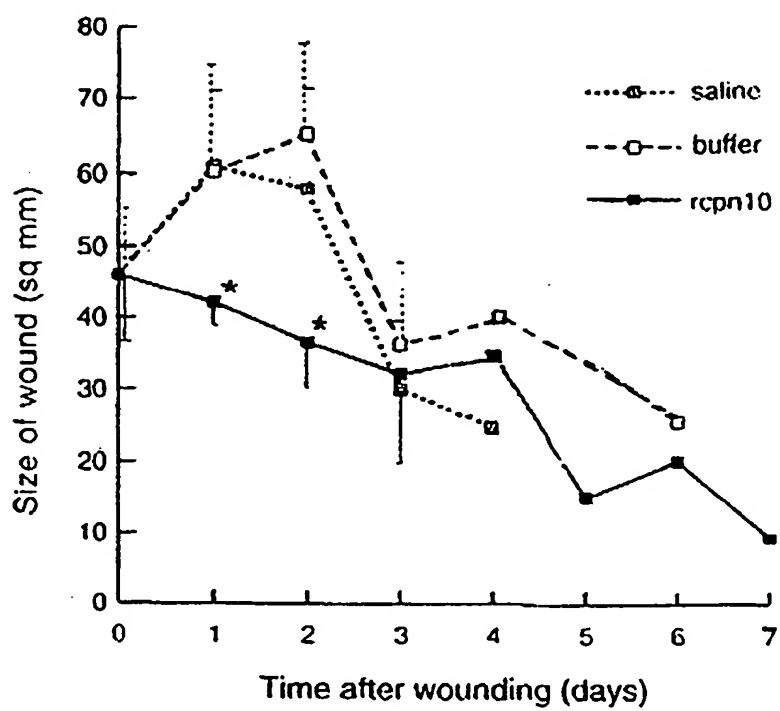


FIG 12

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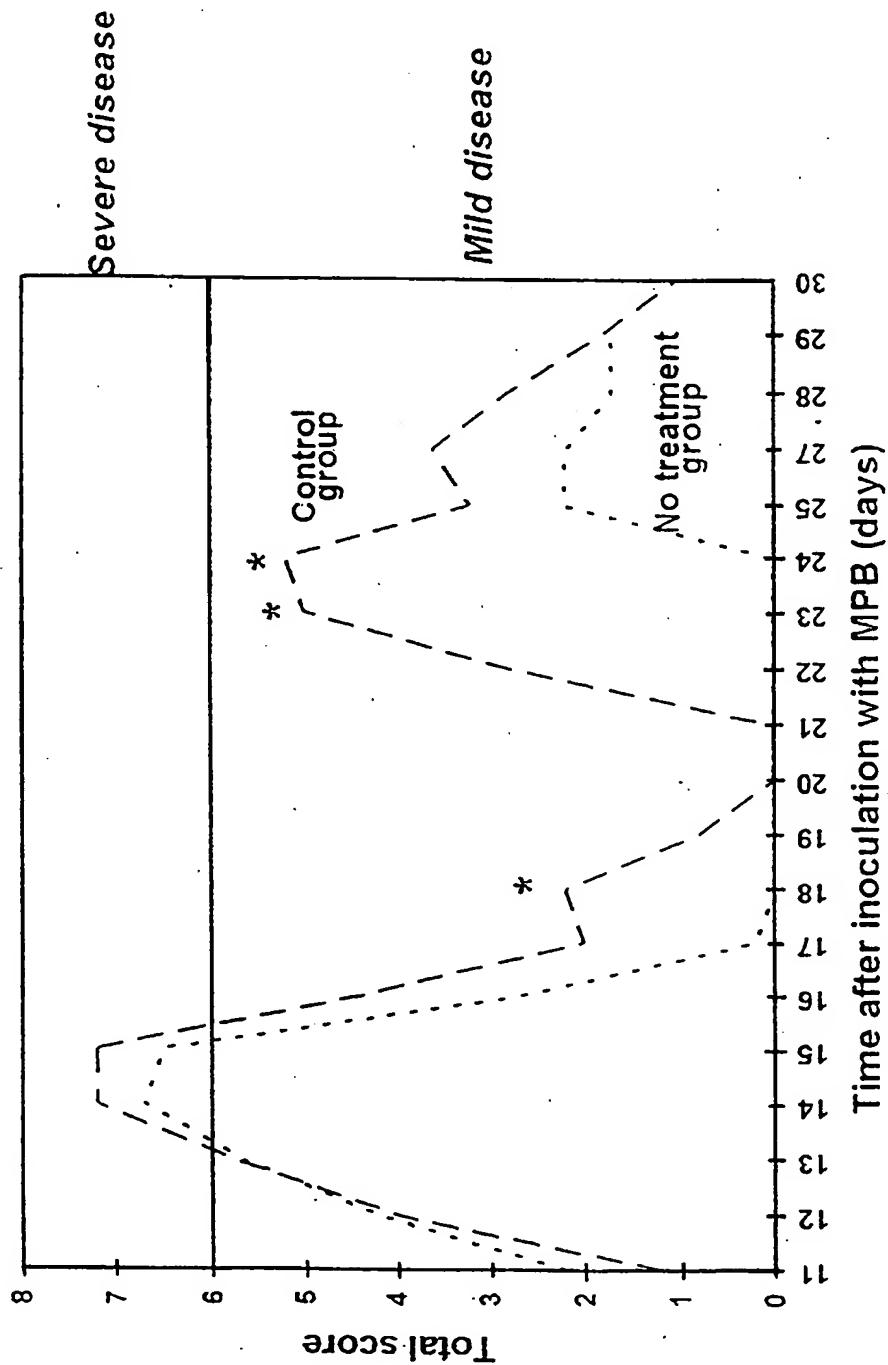


FIG 13



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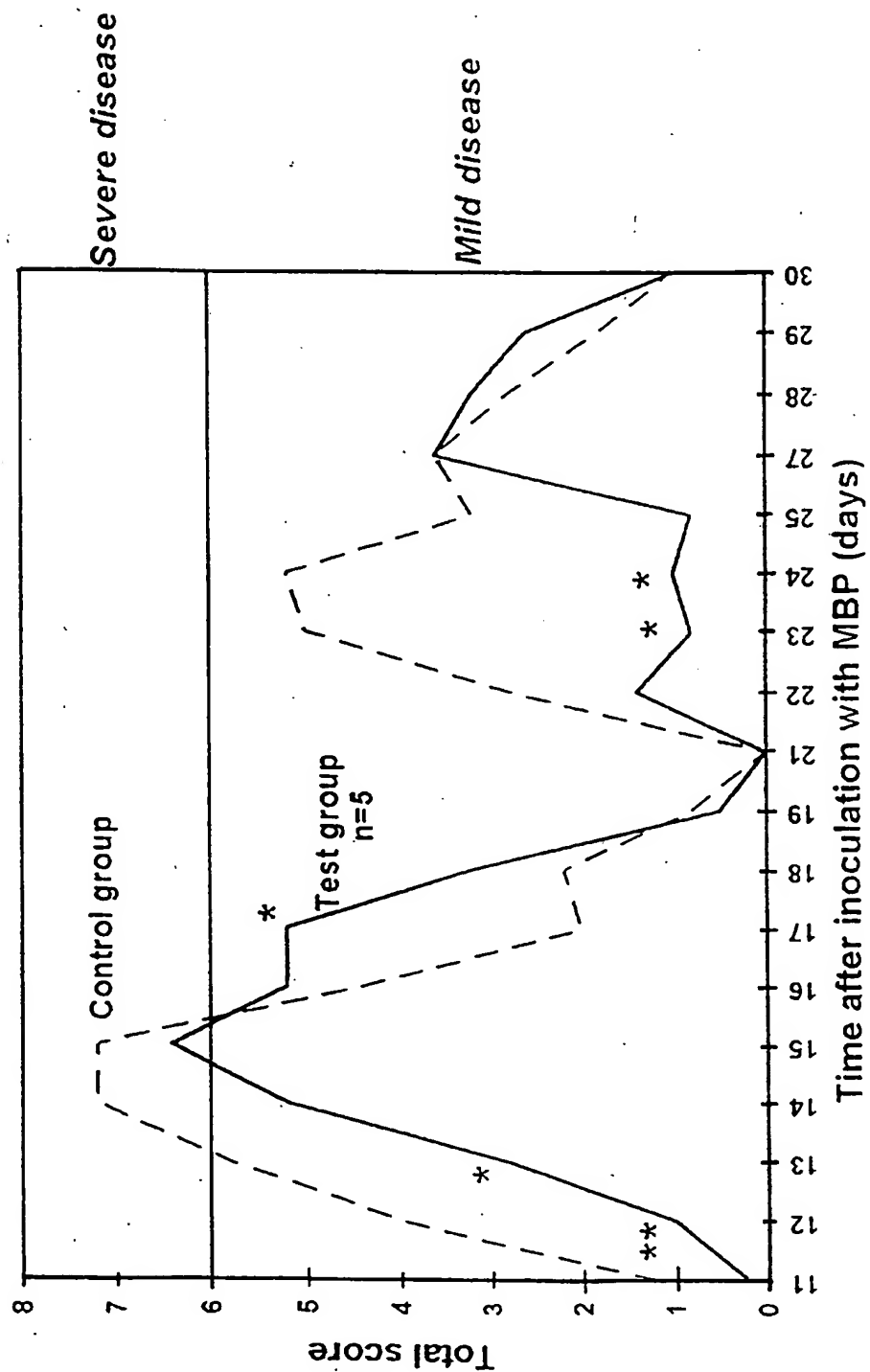


FIG 14

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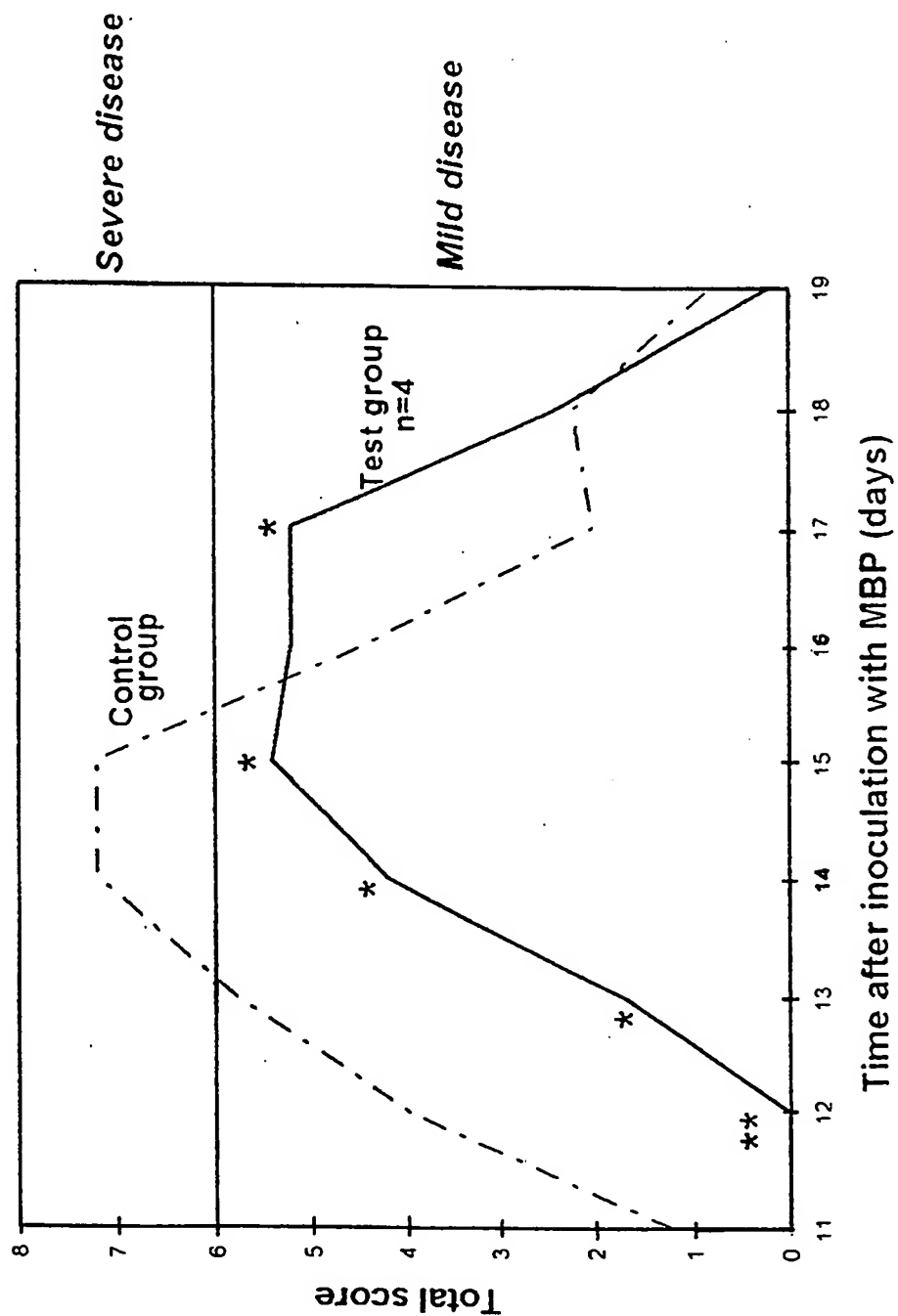
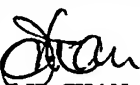


FIG 15

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 94/00740

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl. <sup>6</sup> C07K 14/47, 7/06, C12N 15/12, C12P 21/02, G01N 33/68, A61K 38/08, 38/18, 38/17  According to International Patent Classification (IPC) or to both national classification and IPC					
<b>B. FIELDS SEARCHED</b>  Minimum documentation searched (classification system followed by classification symbols) See KEYWORDS used in electronic data base box below  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched BIOTECHNOLOGY (DERWENT DATABASE) MEDLINE KEYWORDS see electronic data base below  Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT - WPAT, USPM, BIOT, CASM (CHEMICAL ABSTRACTS) )KEYWORDS "CHAPERONIN 10" MEDLING - )OR "EARLY PREGNANCY OR )PREGNANCY FACTOR"  STN INTERNATIONAL - The following protein subsequences: KPPLP OR EKSQG					
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>					
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.			
P,X	European Journal of Biochemistry volume 222 pages 551-560 (1994) Cavanagh Alice C and Morton Halle "The purification of early pregnancy factor to homogeneity from human platelets and identification as chaperonin 10". See specially first column of page 556 and second column of page 558.	1-11			
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.         </div> <div> <input checked="" type="checkbox"/> See patent family annex.         </div> </div>					
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;">           * Special categories of cited documents :             "A" document defining the general state of the art which is not considered to be of particular relevance            "E" earlier document but published on or after the international filing date            "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)            "O" document referring to an oral disclosure, use, exhibition or other means            "P" document published prior to the international filing date but later than the priority date claimed         </td> <td style="width: 33%; vertical-align: top;">           "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention            "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone            "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art            "&amp;" document member of the same patent family         </td> <td style="width: 33%;"></td> </tr> </table>			* Special categories of cited documents :  "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
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Date of the actual completion of the international search 9 March 1995 (09.03.95)		Date of mailing of the international search report 16 Mar 1995 (16.03.95)			
Name and mailing address of the ISA/AU  AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA  Facsimile No. 06 2853929		Authorized officer   J.H. CHAN  Telephone No. (06) 2832340			

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 94/00740

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
P,X	Hepatology volume 20, no. 5 (1994) pages 1294-1302, Quinn Kathryn A. et al "Early Pregnancy Factor in Liver regeneration after partial hepatectomy in rats : relationship with chaperonin 10". See figure 4 table 1 pages 1295, 1298 to 1301	1-11, 12, 15-20
P,X	Febs Letters volume 337 pages 152-156 (1994) Ryan Michael T et al "Isolation of a cDNA clone specifying rat chaperonin 10, a stress-inducible mitochondrial matrix protein synthesised without a cleavable presequence". See figure 1	11
P,X	Biochemica et Biophysica Acta volume 1219, pages 187-190 (1994) Chen Jason J. et al "Isolation, sequence analysis and characterization of a cDNA encoding human chaperonin 10". See figures 1 and 2	11
P,X	Biochemica et Biophysica Acta volume 1218, pages 478-480 (1994) Monzini Nicoletta et al "Identification and cloning of human chaperonin 10 homologue". See figure 3	11
P,X	DNA Sequence - Journal of DNA sequencing and Mapping volume 3 pages 291-295 Pilkington Stephanie J and Walker John E "Complementary DNA sequence of bovine Cpn 10 (Hsp10), a chaperone protein from mitochondria". See figures 1 and 2	11
P,X	Biochemica et Biophysica Acta volume 1164 pages 219-222 (1993) Hartman Dallas J. et al "The complete primary structure of rat chaperonin 10 reveals a putative 8 $\alpha$ nucleotide-binding domain with homnology to p21 ras". See figures 2 and 3	11
X	Proceeding of National Academy of Science USA volume 89 pages 3394-3398 (1992) Hartman Dalls J. et al "Identification of a mammalian 10-kDA heat shock protein, a mitochondrial chaperonin 10 homologue essential for assisted folding of trimeric ornithine transcarbamoylase in vitro". See figures 5	11
P,X	The Journal of Biological Chemistry volume 265 no. 43 (1994) pages 26858-26864 Dickson Ramona et al "Cloning, expression and purification of a functional nonacetylated mannalian into mitochondrial chaperonin 10". See figure 1	11
A	AU,B, 10869/88 University of Queensland 30 June 1988 (30.06.88)	1-10
A	Clinical Experimental Immunology volume 80 (1990) pages 100-108 Quinn K.A et al "Monoclonal antibodies to early pregnancy factor perturb tumour cell growth".	1-10
A	Cancer Immunology and Immunotherapy (1992) volume 34 pages 265-271 Quinn K.A. and Morton H. "Effect of monoclonal antibodies to early pregnancy factor (EPF) on the in vivo growth of transplantable murine tumours".	1

**Information on patent family members**

**PCT/AU 94/00740**

Patent Document Cited in Search Report		Patent Family Member			
AU	10869/88	EP	345260	WO	8804779
<b>END OF ANNEX</b>					

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